THE 14-3-3 PROTEIN TRANSLATES THE Na⁺,K⁺-ATPASE α₁-SUBUNIT PHOSPHORYLATION SIGNAL INTO BINDING AND ACTIVATION OF PHOSPHOINOSITIDE 3-KINASE DURING ENDOCYTOSIS*

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Running Title: 14-3-3/Na,K-ATPase interactions during endocytosis

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Clathrin-dependent endocytosis of Na⁺,K⁺-ATPase molecules in response to G protein-coupled receptor signals is triggered by phosphorylation of the α-subunit and the binding of phosphoinositide 3-kinase. In this study, we describe a molecular mechanism linking phosphorylation of Na⁺,K⁺-ATPase α-subunit to binding and activation of phosphoinositide 3-kinase. Co-immunoprecipitation studies as well as experiments using confocal microscopy revealed that dopamine favored the association of 14-3-3 protein with the basolateral plasma membrane, and its colocalization with the Na⁺,K⁺-ATPase α-subunit. The functional relevance of this interaction was established in opossum kidney (OK) cells expressing a 14-3-3 dominant negative mutant, where dopamine failed to decrease Na⁺,K⁺-ATPase activity and to promote its endocytosis. The phosphorylated Ser-18 residue within the α-subunit N-terminus is critical for 14-3-3 binding. Activation of phosphoinositide 3-kinase by dopamine during Na⁺,K⁺-ATPase endocytosis requires the binding of the kinase to a proline-rich domain within the α-subunit, and this effect was blocked by the presence of a 14-3-3 dominant negative mutant. Thus, the 14-3-3 protein represents a critical linking-mechanism for recruiting phosphoinositide 3-kinase to the site of Na⁺,K⁺-ATPase endocytosis.

Regulation of Na⁺,K⁺-ATPase activity by hormones within the renal tubule epithelial cells provides control for transepithelial sodium transport and thereby urinary sodium excretion during salt loading or deprivation (1). During its regulation, the Na⁺,K⁺-ATPase molecule shuttles between the plasma membrane and intracellular compartments in response to hormones (2). Thus, a balance between the increase or decrease in the number of copies within the plasma membrane determines the cell Na⁺,K⁺-ATPase activity. The responses to different agonists regulating the intracellular distribution of Na⁺,K⁺-ATPase are tissue specific (3), and in renal tubule cells the direction of the physiological response (increase vs decrease) in response to hormones that influence urine sodium excretion appears to depend largely on the concentration of intracellular sodium (4,5).

A complex intracellular signalling network is responsible for the removal or insertion of new molecules within the plasma membrane. In the rodent renal epithelia, phosphorylation of Na⁺,K⁺-ATPase α₁-subunits (at Ser-18) that are present at the plasma membrane is necessary for dopamine (DA)-induced endocytosis (6-8) and at Ser-11 for parathyroid hormone (PTH)-induced endocytosis (9). In contrast, recruitment of new molecules to the plasma membrane in response to angiotensin II (10) or serotonin (11) requires the phosphorylation of Na⁺,K⁺-ATPase molecules (Ser-11/Ser-18) present within endosomes. DA-mediated phosphorylation of the α₁-subunit and Na⁺,K⁺-ATPase endocytosis requires activation of the PKC-ζ isof orm, whereas serotonin- and angiotensin II-dependent increase in Na⁺,K⁺-ATPase activity requires activation of the PKC-β isof orm (11,12). Nonetheless, phosphorylation in either circumstance does not change the catalytic properties of the enzyme (7,13), but constitutes a triggering factor essential for initiating their recruitment into clathrin vesicles and traffic to their final destination.

Endocytosis of Na⁺,K⁺-ATPase molecules in response to DA in renal epithelial cells is initiated by AP-2 binding to the Na⁺,K⁺-ATPase α₁-subunit Tyr-537 residue (14) and clathrin recruitment (15). Endocytosis and binding of AP-2 to the α₁-subunit requires the activation of
phosphoinositide 3-kinase (PI 3-kinase) (16,17). This process is triggered by phosphorylation of the Na⁺,K⁺-ATPase α₁-subunit and binding of the PI 3-kinase (class IA) p85α regulatory subunit (SH3 domain) to a proline-rich motif (PRD) within the α₁-subunit N-terminus upstream of the PKC-phosphorylation site (17). The mechanism that translates phosphorylation of the α₁-subunit Ser-18 residue into binding of PI 3-kinase to the PRD is not yet understood.

Structural studies using analogous comparisons between the Na⁺,K⁺-ATPase α₁-subunit and the tertiary structure of the skeletal muscle sarcoplasmic reticulum Ca²⁺-ATPase α₁-subunit (they share several common structural/functional features) revealed a structurally exposed PRD motif on the molecule N-domain (18,19). This observation may suggest that phosphorylation of the α₁-subunit at Ser-18 might not be required for inducing a structural conformation of the Na⁺,K⁺-ATPase α₁-subunit N-terminus in order to make the PRD accessible for its binding to the PI 3-kinase, but rather to facilitate its interaction with a linker that would make, instead, the PI 3-kinase available to the PRD motif.

The 14-3-3 proteins represent an important model of scaffolding proteins turning serine phosphorylated residues within target proteins into recruitment modules linking the components of diverse cellular signaling networks during a functional response (20-22). Because 14-3-3 proteins are targeted primarily to phosphorylated serine residues, and because of their ability to interact with the PI 3-kinase (23-25), we hypothesized that 14-3-3 proteins may provide the linking mechanisms guiding the PI 3-kinase to the PRD within the α₁-subunit N-terminus after it has been phosphorylated at the Ser-18 residue, thus initiating subunits’ endocytosis.

**MATERIAL AND METHODS**

**Reagents** - The 14-3-3 ε-isofrom wild type and negative mutant (Δ208-255) were kindly provided by Dr T.-A. Sato (Columbia University, New York, NY). The Na⁺,K⁺-ATPase antibody used for immunoprecipitation was a gift of Dr Mercer (Washington University, Saint Louis, MO), and the antibody used for Western blots was a gift of Dr M. Caplan (Yale University, New Haven, CT). A monoclonal antibody against PI 3-kinase was purchased from Transduction Laboratories, and a polyclonal antibody (Z-8) was from Santa Cruz Biototechnology, Inc. The antibody against 14-3-3 (K-19) was purchased from Santa Cruz Biototechnology Inc. Fluorescent labeled antibodies (Alexa dyes) were purchased from Molecular Probes.

**Cell culture and transfection** - Experiments were performed in OK cells expressing stably the rodent Na⁺,K⁺-ATPase α₁-subunit wild type (13) or carrying a green fluorescent tag in its α₁-subunit as described previously (14). OK cells stably transfected with the rodent wild-type α₁-subunit were transiently transfected with plasmids bearing either 14-3-3 wild type or a deletion mutant (26). The cells at 90% confluence in 10 cm dishes were exposed for 24 h to the mixture of 25 µl LipofectAmine 2000 and 10 µg plasmid, preincubated according to Gibco/Invitrogen protocol. Mutations in the PKC phosphorylation site (S11A and S18A), aminoacid deletions, and stable expression of the Na⁺,K⁺-ATPase α₁-subunit were performed as described previously (8).

**Preparation of proximal tubule cells** - PCT cells were obtained as described (27) from Sprague-Dawley rats weighing 150-200 grs. Briefly, homogenates from the kidney outermost cortex were minced and incubated with 0.7 mg/ml collagenase A (Roche Diagnostics GmbH, Manheim, Germany) in 10 ml Dulbecco’s modified Eagle’s medium, DMEM (Life Technologies, Paisley, Scotland). The incubation lasted for 20 min at 37°C, and the solution was continuously exposed to 95% O₂/5% CO₂ during this period. After pouring the material through graded sieves, the cell suspension consisting mostly of PCT cells was washed (4x, in DMEM) and finally resuspended in Hanks’ medium (Life Technologies, Paisley, Scotland) to yield a protein concentration of approximately 1-3 mg/ml. The experiments were performed immediately after preparation.

**Preparation of basolateral membranes** - Basolateral membranes were obtained from PCT cells using a Percoll (Amersham Pharmacia Biotech, AB, Uppsala, Sweden) gradient centrifugation according to Hammond et al. (28). In brief, postnuclear supernatants were centrifuged at 20000 g for 20 min at 4°C. The yellow layer obtained was resuspended in homogenisation buffer and further centrifuged at 48000 g for 30 min at 4°C. The pellet was resuspended in 1 ml homogenisation buffer and 0.2 ml of Percoll was added. The suspension was gently mixed and centrifuged at 48000 g for 30 min at 4°C.

**Biotin labeling** - Transiently transfected cells after 24 h were transferred to Hanks solution for 30 min, and then incubated for 5 min with 1 µM DA or vehicle at room temp. Incubation was stopped on ice, and medium was changed to ice-cold 10 mM Tris-HCl (pH 7.5), 2 mM CaCl₂, 150 mM NaCl,
1.5 mg/ml Sulfo-NHS-Biotin, and the cells were incubated for 1 h at 4°C. Following biotin labelling, the cells were scraped in IP buffer (20 mM Tris, 2 mM EDTA, 2 mM EGTA, 30 mM sodium pyrophosphate, pH 7.3) containing a protease inhibitor cocktail, frozen in liquid nitrogen, thawed rapidly, probe-sonicated twice on ice-water bath, and frozen-thawed again. The cell suspension was centrifuged at 14,000 g at 4°C for 5 min. Supernatants were transferred to clean tubes and 1% Triton X-100 and 0.2% SDS were added. Anti-α1 monoclonal antibody was added and incubated for 1 h at 4°C with end-over-end shaking, then Protein A/G agarose, pre-washed three times with PBS and once with immunoprecipitation buffer containing 1% Triton X-100, was added and incubated overnight. The pellet was washed three times with this buffer containing 1% Triton X-100 and 0.1% SDS, once with 50 mM Tris-Cl (pH 7.4), and finally resuspended in Laemmli sample buffer. Electrophoresis, Western blot using Extravidin, and densitometric analysis were performed as previously described (13).

**Determination of Na,K-ATPase activity -** Na⁺,K⁺-ATPase activity was determined from the ouabain-inhibitable ⁸⁶Rb⁻-transport. To assess the effect of DA, cells were pre incubated at room temperature with 5 µM monensin (Sigma) for 30 min as described by Seri et al. (5), and then with 1 µM DA (5 min) before assay. Measurements of Na⁺, K⁺-ATPase-mediated ⁸⁶Rb⁻-transport were performed as described (13) and Na⁺, K⁺-ATPase activity was expressed as nmol Rb/mg prot/min.

**Determination of PI 3-kinase activity -** After preincubation with DA under different conditions, the cells were transferred to 1.5 ml Eppendorff tubes in the cold, homogenized in 400 µl of lysis buffer [137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 0.5 mM Na₃VO₄, 1% Triton X 100, 10% v/v Glycerol, 20 mM TrisHCl, 10 µg/ml Leupeptin, 0.2 mM PMSF, 10 mM NaF, 10 mg/ml Aprotinin] and solubilized by passing through needles (27G x ¾”) for 20 times and by stroking with a pestle for 30 seconds. After centrifugation at 800xg 10min, the supernatant was collected and 500 µg of protein (in 1 ml) was incubated with an antibody against the Na⁺, K⁺-ATPase α₁-subunit (gift Dr Mercer) by end-over-end rotating overnight. Next morning 40 µl A/G agarose beads (Phar-macia) were added and incubated for 2 hrs by end-over-end rotating. The immune complex was washed 3 times with lysis buffer, 2 times with washing buffer 2 [137 mM NaCl, 0.1 M TrisHCl, 10 µg/ml Leupeptin, 0.2 mM PMSF, 10 mM NaF, 10 mg/ml Aprotinin], once with washing buffer 3 [0.15 M NaCl, 1 mM EDTA, 10mM TrisHCl, 10 µg/ml Leupeptin, 0.2 mM PMSF, 10 mM NaF, 10 mg/ml Aprotinin], once with washing buffer 4 [20 mM Hapes, 1 mM DTT, 5 mM MgCl₂, 10 µg/ml Leupeptin, 0.2 mM PMSF, 10 mM NaF, 10 mg/ml Aprotinin] and then resuspended in 20 µl kinase assay buffer [20 mM β-Glycerophosphate, 5 mM Na-Pyrophosphate, 50 mM TrisHCl, 1 mM DTT]. The PI 3-K activity in the immunoprecipitate was assessed directly on the protein A/G agarose beads as described (29). The reaction was initiated by addition of 30 µl of reaction mix [20 µl buffer [12.5 µM ATP, 7.6 mM MgCl₂, 20 mM β-Glycerophosphate, 5 mM Na-Pyrophosphate, 30 mM NaCl, 1 mM DTT, 4µCi [γ⁻³²P]ATP] plus 40 µg lipids (Avanti Biochemicals, Birmingham, AL) in 10 µl cholate buffer (10 mg sodium cholate/ 1 ml Kinase Assay buffer)]. The pellets were incubated for 15 min at room temperature, and the reaction was terminated by sequential addition of 20 µl HCl and 160 µl chloroform/methanol (1:1, vol/vol) with vigorous vortexing. After centrifugation at 14000 rpm 5 min, 80 µl of the lower phase was spotted on aluminium-backed Silica Gel TLC plates (MERCK, Germany). The lipids were resolved by chromatography in methanol/CHCl₃/ammonia/H₂O (47:34:12.5:6.5). The dot corresponding to phosphatidylinositol 3-phosphate was analyzed by autoradiography and quantitated using phosphoimaging.

**Immunoprecipitation -** OK cells grown in Petri dishes (10 cm) were incubated in Hanks’ medium for 30 min at 25°C prior to incubation in the presence or absence of 1 µM DA for the times indicated above at 23°C. Thereafter, incubation solutions were replaced by immunoprecipitation buffer [in mM, 100 NaCl, 50 Tris-HCl, 2 EGTA, 1 PMSF, 5 mg/ml of protease inhibitors (aprotinin, leupeptin, antipain), 1% Triton X-100 (pH 7.5)] and the samples were transferred to ice. The cells were disrupted by homogenisation with a motor pestle homogenizer. Immunoprecipitation of the Na⁺, K⁺-ATPase α₁-subunit, PI 3-kinase and 14-3-3 was performed as previously described (13). In brief, aliquots (500 µg protein) were incubated overnight at 4°C with 70 µl of a Na⁺, K⁺-ATPase antibody (gift Dr Mercer), 6 µg of a polyclonal antibody raised against the PI 3-kinase p85α subunit (Z-8, Santa Cruz, CA), or with 5 µg of 14-3-3 antibody (K-19, Santa Cruz, CA), and the simultaneous addition of excess protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). Protein content was determined according to Bradford (30). Samples were analysed by SDS/PAGE using the Laemmli buffer system (31). Proteins were transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia AB), and Western blots were performed.
using an antibody against the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit and developed with an ECL Plus (Amersham Pharmacia Biotech AB) detection kit.

**Microscopy** - These experiments were performed using OK cells stably transfected with the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit carrying a GFP-tag in the NH\textsubscript{2} terminus. The presence of the tag does not affect the intrinsic properties of the enzyme or its regulation by dopamine signals (14,32). OK cells were incubated in the presence or absence of 1 \(\mu\text{M}\) DA for 2 min at 23\(^\circ\text{C}\). Incubation was terminated by fixation of the cells with 4\% formaldehyde in PBS for 10 min at room temperature. After rinsing twice with PBS, cells were transferred to acetone (-20\(^\circ\text{C}\)) for 5 min and then quenched with PBS (containing 1\% BSA) for 30 min. Staining with primary \([14-3-3 (1:100)]\) and secondary fluorescent labelled antibody \((1:100)\) was performed at room temperature for 1 h. After rinsing with PBS the coverslips were mounted (SlowFade Light, Molecular Probes, Eugene, OR) and examined using a confocal microscope (Leica TCS SP2, Leica Lasertechnik GmbH, Heidelberg, Germany).

**Statistics** - Comparison between two experimental groups was made with the nonpaired Student’s \(t\)-test. \(p < 0.05\) was considered significant. In all figures bars indicate mean \(\pm\) SD.

**RESULTS AND DISCUSSION**

Phosphorylation of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit in response to G protein-coupled receptor (DA) stimulation is central for organizing, in time and space, a variety of signals that ultimately will lead to the endocytosis of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase molecules. Among those signals, binding (to a proline-rich motif within the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit) and activation of PI 3-kinase activity is of outmost relevance for recruiting recognition molecules (adaptins) and clathrin formation (15,17), as well as recruiting dynamin for clathrin vesicle fission (33). Phosphorylation of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit at ser-18 is critical for PI 3-kinase binding, but the molecular mechanisms of this interaction remain unknown. The fact that 14-3-3 proteins can form dimers (20) thereby creating interactive modules potentially linking serine phosphorylated residues with diverse signalling molecules [including PI 3-kinase (23-25)] prompted us to study the possibility that its association with the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit may guide the PI 3-kinase regulatory subunit (p85\(\alpha\)) to the proline-rich domain upstream of the Ser-18 phosphorylation site, and regulate Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activity and subunit endocytosis.

Because there are numerous 14-3-3 isoforms we examined their presence in PCT cells isolated from rat kidney by Western blotting using an antibody that recognizes all the isoenzymes (34). The 14-3-3 was present in PCT cells and high-speed centrifugation of PCT cells homogenate revealed that most of the protein is present in the cytosol (Fig. 1A). Whereas a negligible amount was associated with the basolateral membranes fraction, the presence of DA induced a time-dependent increase in the abundance of 14-3-3 within this compartment (Fig. 1B). Additionally, confocal images of OK cells revealed the presence of 14-3-3 mostly in the cytosol in non-treated cells (V: vehicle) (Fig. 1C), whereas in the presence of DA there is and increased 14-3-3 immunofluorescence associated with the plasma membrane (arrows) (Fig. 1C, D: dopamine). Furthermore, support for a direct association of 14-3-3 with the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase molecules at the plasma membrane in response to DA signals was obtained in coimmunoprecipitation assays. DA treatment is associated with increased Na\textsuperscript{+}, K\textsuperscript{+}-ATPase immunoreactivity in the material immunoprecipitated with a 14-3-3 protein antibody (Fig. 2A, left panel), and in the reciprocal experiment increased 14-3-3 is observed in the material immunoprecipitated with a Na\textsuperscript{+}, K\textsuperscript{+}-ATPase antibody (Fig. 2A, right panel). These results strongly suggest that in response to DA signals the 14-3-3 becomes associated with basolateral plasma membrane where it binds to the phosphorylated Ser-18 residue in the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit.

Although 14-3-3 binds to proteins at a consensus motif (RSXPXSP) where pS is a phosphorylated serine critical for triggering its kinase binding, but the molecular mechanisms of this interaction remain unknown. The fact that 14-3-3 proteins can form dimers (20) thereby creating interactive modules potentially linking serine phosphorylated residues with diverse signalling molecules [including PI 3-kinase (23-25)] prompted us to study the possibility that its association with the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase \(\alpha_1\)-subunit may guide the PI 3-kinase regulatory subunit (p85\(\alpha\)) to the proline-rich domain upstream of the Ser-18 phosphorylation site, and regulate Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activity and subunit endocytosis.
Ser-18 residue. Mutation in another adjacent PKC phosphorylation site (S11) did not affect the interaction between Na⁺,K⁺-ATPase and 14-3-3 triggered by DA. These results indicate that Ser-18 within the rat Na⁺,K⁺-ATPase α₁-subunit is a likely binding site for 14-3-3 in response to DA. A weak interaction between these proteins can also be noticed in non-stimulated cells, and this association was not entirely absent in cells expressing the α₁-subunit carrying the S18A mutation or the 1-26 deletion, suggesting the possibility that other 14-3-3 binding sites might be present within the Na⁺,K⁺-ATPase α₁-subunit which, however, are not under DA control.

Although the experimental data obtained strongly suggested a structural interaction between the 14-3-3 and the Na⁺,K⁺-ATPase α₁-subunit, the following experiments were designed to examine whether such interaction had also functional relevance, i.e., if it is necessary for the regulation of Na⁺,K⁺-ATPase activity and the removal of active units from the plasma membrane in response to DA. OK cells were transiently transfected with wild type 14-3-3 or a deletion mutant (Δ208-255) of this protein lacking essential aminoacids (in bold: Lys-49, Arg-59, Arg-127, Tyr-128, Trp-228, Asn-173, Lys-120, Asn-224, Leu-216, Ile-217, Leu-220) within the phosphopeptide interactive domain (26,35). DA decreased Na⁺,K⁺-ATPase activity in non-transfected cells, mock-transfected cells and in OK cells transfected with the wild type 14-3-3, whereas it failed to do so in OK cells transiently expressing the 14-3-3 mutant (Fig. 3A). Moreover, in OK cells expressing the mutant form of 14-3-3, DA failed to promote the endocytosis of Na⁺,K⁺-ATPase molecules (Fig. 3B). These results further suggest a functional interaction between 14-3-3 protein and the Na⁺,K⁺-ATPase α₁-subunit. Whereas the 14-3-3 mutant employed may have limited its ability to interact with the phosphorylated Na⁺,K⁺-ATPase α₁-subunit, we can not exclude the possibility that this mutant did also affect its recognition motif with other proteins involved in the Na⁺,K⁺-ATPase regulatory process, such as the PI 3-kinase.

It has been demonstrated in several cell systems that the 14-3-3 protein binds to PI 3-kinase (23-25) and thereby affects various physiological functions. Therefore, OK cells were incubated with or without DA and the presence of PI 3-kinase (p85α-subunit) was examined in the immunoprecipitated material with a 14-3-3 antibody (Fig. 4A). Indeed, cells incubated with DA demonstrated a significant increase in PI 3-kinase associated with 14-3-3 compared to vehicle-treated cells. To establish whether the interaction of PI 3-kinase with the Na⁺,K⁺-ATPase requires 14-3-3, we next examined their colocalisation in response to DA in OK cells transiently expressing the 14-3-3 wild type or mutant (Fig. 4B). In the presence of DA, there was an increased colocalization of the Na⁺,K⁺-ATPase α-subunit and PI 3-kinase in nontransfected and mock-transfected cells, and in cells transfected with the wild type 14-3-3, but not in cells expressing transiently the 14-3-3 mutant. These data strongly indicate that the 14-3-3 represents a link between the phosphorylated Na⁺,K⁺-ATPase α-subunit and binding of PI 3-kinase to its proline-rich domain within the N-terminus. In addition, transient expression of the 14-3-3 negative mutant also prevented the increase in PI 3-kinase activity associated with the binding of the Na⁺,K⁺-ATPase α-subunit to the p85α regulatory subunit (Fig. 5). We do not know yet the nature of the interaction between 14-3-3 and the PI 3-kinase. It is possible that such interaction may involve either of the kinase subunits (p85α or p110) or an alternative regulatory protein.

Binding of 14-3-3 to the Na⁺,K⁺-ATPase α₁-subunit may not be limited to providing an anchor for the PI 3-kinase. Thus, 14-3-3 binding to phosphorylated serine residues modulates the action of protein phosphatases upon those residues with direct functional consequences in the physiological responses. For example, dephosphorylation of NHE1 and HERG-K⁺ channel was protected by the binding of 14-3-3, thereby resulting in stimulation of NHE1 activity and prolonged HERG-K⁺ channel activation by adrenergic stimulation (37), as well as dephosphorylation of BAD and regulation of its pro-apoptotic function (38). Because phosphorylation of the Na⁺,K⁺-ATPase α₁-subunit is essential for triggering its endocytosis, it is likely that the action of 14-3-3, in addition to facilitating the binding of PI 3-kinase, may also protect Ser-18 from being dephosphorylated by the action of protein phosphatases. Indeed, we previously reported (15) that Na⁺,K⁺-ATPase endocytosis was not blocked by dephosphorylation of the α₁-subunit but rather through an increase in inositol hexakisphosphate, which prevents the association of adaptor proteins with the Na⁺,K⁺-ATPase. Thus, the presence of 14-3-3 protecting the Ser-18 phosphorylation site could prevent the phosphatase activity associated with the PP2A that constitutively binds to the Na⁺,K⁺-ATPase α₁-subunit (39).

These results provide the identity of a new signaling partner, the 14-3-3 protein, that interacts with the Na⁺,K⁺-ATPase molecule at the plasma membrane (Fig. 6). This association requires phosphorylation of the Na⁺,K⁺-ATPase α₁-subunit N-terminus and it permits the binding of PI 3-kinase to a proline-rich domain located upstream of the phosphorylation site. Thus, 14-3-3 is an
essential part of the signaling network regulating Na⁺,K⁺-ATPase activity and endocytosis in response to G protein-coupled receptor ligands, a process that is essential for controlling kidney tubule Na⁺,K⁺-ATPase activity in response to natriuretic hormones.

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FOOTNOTES

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1The abbreviations used are: DA, dopamine; PKC, protein kinase C; PI 3-kinase, phosphoinositide 3-kinase; AP-2, adaptor protein-2; PCT, proximal convoluted tubules; OK, opossum kidney; NH1, Na+/H+ exchanger isoform-1; HERG, *human ether-a-go-go related gene*; BAD, Bcl2-antagonist of cell death.

FIGURE LEGENDS

Fig. 1. Presence of 14-3-3 in PCT cells. A, Homogenates (H), cytosol (C), total membranes (M), and basolateral membranes (BLM) prepared from PCT cells were separated on SDS-PAGE (31) and Western blot analysis was performed using a 14-3-3 antibody (1:500). Equal amounts of protein (10 µg) was analysed in each lane. B, PCT cells were incubated with 1 µM DA at 23ºC for different periods of time, and the presence of 14-3-3 in basolateral membranes determined by Western blot. Quantitative analysis of four experiments performed independently is presented. Inset: representative Western blot of 10 µg BLM analysed in each lane. C, Confocal images of 14-3-3 (arrows) in fixed OK cells previously treated with (D) or without (V) 1 µM DA for 2 min at 23ºC. The experiment was repeated twice and several cells were analysed in each experiment.

Fig. 2. A, Interaction of 14-3-3 with the Na+,K+-ATPase. Homogenates (500 mg) from PCT cells previously incubated in the presence (DA) or absence (V: vehicle) of 1 µM DA for 2 min at 23ºC, were incubated with either a Na+,K+-ATPase (right panel) or 14-3-3 (left panel) (5 µg) antibody. The immunoprecipitated material was analysed by Western blot with either Na+,K+-ATPase (1:1000) or 14-3-3 (1:500) antibody. A representative Western blot of five experiments is shown. B, Coimmunoprecipitation of the Na+,K+-ATPase with 14-3-3 protein was performed exactly as described above (A) in OK cells stably transfected with the Na+,K+-ATPase bearing the mutation Ser 11→Ala (S11A), Ser 18→Ala (S18A) or deletion of the first 26 amino acids (∆26) in the α1-subunit, as described (8). Upper panel: representative Western blot. Lower panel: quantitative analysis of 2 experiments.

Fig. 3. DA-dependent inhibition of Na+,K+-ATPase activity and subunit endocytosis requires 14-3-3 proteins. A, Na+,K+-ATPase activity (% of control) in OK cells transiently expressing the wild type (14-3-3-WT) or a deletion mutant (14-3-3-M) of 14-3-3 proteins. A, Na+,K+-ATPase activity (% of control) in OK cells transiently expressing the wild type (14-3-3-WT) or a deletion mutant (14-3-3-M) of 14-3-3. NT: non-transfected and mock (Lipofectamine-treated) OK cells. Each bar represents the mean of 3 experiments performed in triplicate. B, Na+,K+-ATPase abundance at the plasma membrane was examined by cell surface biotinylation in transiently transfected OK cells as described in A. OK cells were incubated with 1 µM DA for 2 min at 23ºC. A representative Western blot of three experiments is shown.

Fig. 4. A, Interaction between PI 3-kinase and 14-3-3 protein. Homogenates (500 mg) from PCT cells that were previously incubated in the presence (DA) or absence (V: vehicle) of 1 µM DA for 2 min at 23ºC, were incubated with a 14-3-3 (5 µg) antibody. The immunoprecipitated material was analysed by Western blot with a PI 3-kinase (1:1250) antibody. B, Association of PI 3-kinase with the Na+,K+-ATPase α1-subunit in OK cells transiently expressing the wild type (14-3-3-WT) or deletion mutant (14-3-3-M) of 14-3-3. NT: non-transfected and mock (Lipofectamine-treated) OK cells. Left panel, representative Western blot; Right panel, quantitation of 3 experiments.
Fig. 5. PI 3-kinase activity was determined in the material immunoprecipitated with a Na⁺,K⁺-ATPase α-subunit antibody from cells transfected with the vector (mock) or wild type (14-3-3-WT), or a deletion mutant (14-3-3-M) of 14-3-3. Representative experiment (Upper panel) and the quantitative analysis of 3 independent experiments is depicted (Lower panel).

Fig. 6. Schematic representation of the molecular organization of different signaling molecules during Na⁺,K⁺-ATPase α₁-subunit endocytosis in response to G protein-coupled receptor signals. The N-terminus of the α₁-subunit represents a scaffolding organizing the assembly of signaling molecules necessary for Na⁺,K⁺-ATPase endocytosis. The signal is initiated by phosphorylation of the Ser-18 residue (KKS¹⁸KK) followed by binding of 14-3-3 and locking of PI 3-kinase (via its SH3 domain) with the proline-rich domain (PP⁷⁸PTTP). Binding and activation of PI 3-kinase (PI 3-k) serves several purposes, e.g.: production of phosphatidylinositol 3-phosphate that is necessary for increasing the affinity of AP-2 (µ2) subunit to a tyrosine rich motif (Y⁵³⁷LEL in the α₁-subunit); and recruiting dynamin, which is necessary for fission of clathrin-coated pits at the site of Na⁺,K⁺-ATPase endocytosis.
Figure 1
Figure 2
Figure 3
**Figure 4**

### A

- **IP:** 14-3-3
- **WB:** PI 3-kinase

- **PI 3-K →**
  - V
  - DA

### B

- **IP:** Na,K-ATPase
- **WB:** PI 3-kinase

- **PI 3-K**
  - NT
  - mock
  - 14-3-3 (WT)
  - 14-3-3 (M)

- **Na,K-ATPase**
  - DA, 1 µM
  - - - - + - +

- PI 3-kinase abundance (% change)
  - NT
  - mock
  - 14-3-3 (WT)
  - 14-3-3 (M)
Figure 5

mock 14-3-3

14-3-3 (WT) 14-3-3 (M)

PI3P

Origin

DA, 1 µM - + - - +

0

50

100

PI3-kinase activity (% change)

mock 14-3-3 (WT) 14-3-3 (M)

-50

0

50

100

Average PI3-kinase activity
Figure 6

Plasma membrane

extracellular

PP$_2$PTTP → PtdIns 3-P → dynamin

KK$^\text{S}$$^\text{P}$$^\text{K}$ → 14-3-3

NH$_2$

Y$^{537}$LEL

AP-2 (μ2)
The 14-3-3 protein translates the Na+\(\text{,K}^{+}\)-ATPase \(\alpha1\)-subunit phosphorylation signal into binding and activation of phosphoinositide 3-kinase during endocytosis

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