Tyrosine 537 within the Na\textsuperscript{+},K\textsuperscript{+}-ATPase \(\alpha\)-Subunit Is Essential for AP-2 Binding and Clathrin-dependent Endocytosis*  

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In renal epithelial cells endocytosis of Na\textsuperscript{+},K\textsuperscript{+}-ATPase molecules is initiated by phosphorylation of its \(\alpha\)-subunit, leading to activation of phosphoinositide 3-kinase and adaptor protein-2 (AP-2)/clathrin recruitment. The present study was performed to establish the identity of the AP-2 recognition domain(s) within the Na\textsuperscript{+},K\textsuperscript{+}-ATPase \(\alpha\)-subunit. We identified a conserved sequence (Y\textsuperscript{537}EL) within the \(\alpha\)-subunit that represents an AP-2 binding site. Binding of AP-2 to the Na\textsuperscript{+},K\textsuperscript{+}-ATPase \(\alpha\)-subunit in response to dopamine (DA) was increased in OK cells stably expressing the wild type rodent \(\alpha\)-subunit (OK-WT), but not in cells expressing the Y537A mutant (OK-Y537A). DA treatment was associated with increased \(\alpha\)-subunit abundance in clathrin vesicles from OK-WT but not from OK-Y537A cells. In addition, this mutation also impaired the ability of DA to inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity. Because phosphor esters increase Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in OK cells, and this effect was not affected by the Y537A mutation, the present results suggest that the identified motif is specifically required for DA-induced AP-2 binding and Na\textsuperscript{+},K\textsuperscript{+}-ATPase endocytosis.

In renal epithelial cells the Na\textsuperscript{+},K\textsuperscript{+}-ATPase is located within the basolateral domain (1) and shuttles between the plasma membrane and intracellular organelles during its regulation by G protein-coupled receptor signals. This process is critical for the regulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity and its availability at the plasma membrane in response to G protein-coupled receptor signals. AP-2 antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Dopamine was purchased from Solvay Pharmaceuticals GmbH (Hannover, Germany). All other reagents were of highest available grade.

Plasmid Construction—To obtain plasmid pCMV.GFP.Na\textsuperscript{+},K\textsuperscript{+}-ATPase we first introduced a Nru\textsubscript{I} site into the 5' untranslated region of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, which is site-directed mutagenesis. The GFP0 cDNA, which lacks the stop codon, was obtained from pB.CMV.GFP0 (13) and was inserted in-frame in pCMVouabain-NruI following digestion with NruI and Clal. Mutants of the un-tagged and the N-terminally GFP-tagged Na\textsuperscript{+},K\textsuperscript{+}-ATPase were created by site-directed mutagenesis by exchanging nucleotides as follows: Y506F (TAC versus TTC), Y525F (TAC versus TTC), Y469F (TAC versus TTC), Y537F (TAC versus TTC), Y537A (TAC versus GCC), Y679F (TAC versus TTC), L499A (CTG versus GCC), and L554A (CTT versus GCC). All mutations were introduced by employing the QuiKChange Mutagenesis Kit from Stratagene, and oligonucleotides bearing the respective nucleotide exchanges were synthesized at Genset. All constructs were verified by DNA sequence analysis.

Cell Culture and Transfection—OK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum, penicillin/streptomycin (100 IU/ml and 100 \(\mu\)g/ml, respectively) and 2 \(\mu\)M glutamine in a 5% CO\textsubscript{2} incubator at 37 °C. Cells were transfected with various expression constructs using the LipofectAMINE technique (LipofectAMINE, Invitrogen) (14, 15). Two days after transfection, cells were harvested and analyzed as described.
Binding of AP-2 to a Na,K-ATPase Tyrosine-based Motif

Potential endocytic sequence within the Na,K-ATPase α1-subunit. Several tyrosine motifs were identified within the N terminus and the cytoplasmic loops between M1-M2 and M4-M5 (left panel). Na,K-ATPase activity was determined in OK cells stably expressing the α1-subunit in which either tyrosine residue was replaced by a phenylalanine (right panel). Cells were incubated with 1 μM DA or vehicle (Hanks' medium) for 5 min at 23 °C. Each bar represents the mean ± S.E. of six determinations performed in triplicate. *, p < 0.05.

FIG. 1. 

Effect of DA on OK cells expressing stably the Na,K-ATPase α1-subunit tagged with GFP. A, OK cells (stably expressing the α1-subunit with open bars) or without (closed bars) GFP were incubated with 1 μM DA or vehicle (Hanks' medium) for 5 min at 23 °C, and Na,K-ATPase activity was determined. Each bar represents the mean ± S.E. of four independent determinations performed in triplicate. **, p < 0.01. B, OK cells transfected with the Na,K-ATPase bearing GFP in its α1-subunit were incubated with DA as indicated in A. Western blot analysis with an antibody against GFP (1:500) was performed in the immunoprecipitated material obtained with an AP-2 antibody. C, Na,K-ATPase α1-subunit abundance was determined by Western blot analysis in CCV from OK cells expressing the Na,K-ATPase α1-subunit bearing GFP. Before CCV preparation, OK cells were treated with DA as described in the legend to A. The data are representative of four experiments.

RESULTS AND DISCUSSION

Using site-directed mutagenesis in intact cells we identified a sequence within the Na,K-ATPase α-subunit that interacts with AP-2 and demonstrated that this interaction is essential for DA-dependent regulation of Na,K-ATPase activity and endocytosis.

Chlorin-dependent endocytosis of Na,K-ATPase molecules in response to G protein-coupled receptor signals requires the interaction of AP-2 with the Na,K-ATPase α-subunit (13, 14). The AP-2 molecules recognize and bind to short consensus motifs present in membrane proteins to be internalized (22) and thereby participate in the recruitment of clathrin to the site of endocytosis. Many AP-2-target interactions involve...
the AP-2 μ-chain binding to a consensus NPXY or YppΦ motif (23), where Y is tyrosine, X is any amino acid, p are preferentially positively charged residues, and Φ is a residue with a bulky hydrophobic chain. Analysis of the Na⁺,K⁺-ATPase α-,subunit sequence revealed several intracellular sites for possible interaction with AP-2 (Fig. 1, left panel), whereas the β-subunit has one consensus motif that is located within the putative transmembrane domain.

The tyrosine residues identified in all those potential AP-2 binding motifs were initially mutated to phenylalanine. This mutation eliminates only the hydroxyl groups but keeps the aromatic characteristic of the amino acid side chain. OK cell lines stably expressing the Na⁺,K⁺-ATPase bearing either of these mutations were generated, and determination of Na⁺,K⁺-ATPase activity (a reflection of endocytosis, Ref. 9) in response to DA was used as read-out. Whereas nonstimulated Na⁺,K⁺-ATPase activity was similar in all groups (mutants and wild type), only OK cells expressing the Na⁺,K⁺-ATPase-Y537F mutant demonstrated a significant, although not complete, reduction in the inhibitory response to DA (Fig. 1, right panel). We studied this motif further by introducing a different mutation, Tyrβ37 → Ala (Y537A); since alanine is an aliphatic amino acid, the mutation, Tyr → Ala should totally eliminate any possible interaction between AP-2 and the amino acid side chain. The experiments were performed in OK cells stably expressing the rat Na⁺,K⁺-ATPase α1-,isoform carrying a GFP tag at the N terminus (α-WT). The presence of this tag did not affect the basal nonstimulated Na⁺,K⁺-ATPase activity (Fig. 2A). Furthermore, DA decreased enzyme activity (Fig. 2A), enhanced the interaction of the α-,subunit with AP-2 (Fig. 2B), and promoted the endocytosis of active molecules in clathrin-coated vesicles (CCV) (Fig. 2C). Introduction of the Y537A mutation (α-Y537A) did not affect the level of α-,subunit expression, as evident from images of the intrinsic GFP fluorescence using confocal microscopy (Fig. 3A) and Western blot analysis using a GFP- or α-subunit antibody (Fig. 3B).

Cells expressing either the wild type or mutated GFP-tagged Na⁺,K⁺-ATPase isoforms both had comparable catalytic activity, determined as the rate of ouabain-sensitive Rb⁺ transport in intact OK cells (Fig. 3C). However, while DA inhibited Na⁺,K⁺-ATPase in OK cells expressing the wild type α-,subunit, it failed to induce a significant change in enzyme activity in OK cells expressing the α-Y537A mutant (Fig. 3C). This mutation, contrary to α-Y537F, completely blocked the inhibitory effect of DA on Na⁺,K⁺-ATPase activity.

Co-immunoprecipitation assays were performed to further establish whether the Y537A mutation has indeed rendered the Na⁺,K⁺-ATPase catalytic α-,subunit unable to recognize AP-2 molecules, thus leading to deficient endocytosis in response to DA (Fig. 3D). Incubation with DA increased the amount of Na⁺,K⁺-ATPase α1-,subunit immunoprecipitated with an AP-2 antibody in α-WT, and, as predicted, this interaction was absent in α-Y537A cells (Fig. 3D, left panel). In another set of experiments, using the same strategy, we demonstrated an increase in AP-2 from the immunoprecipitated material with a Na⁺,K⁺-ATPase antibody in DA-treated α-WT, but not α-Y537A, cells (Fig. 3D, right panel). The sequence identified (Yβ37LEL) is highly conserved among several species.

**TABLE I**

|  | α-WT | α-L499A | α-L554A |
|---|------|---------|---------|
| Vehicle | 10.9 ± 0.7 | 9.6 ± 1.0 | 10.0 ± 0.9 |
| Dopamine, 1 μM | 6.0 ± 0.9⁵ | 6.9 ± 0.9⁵ | 5.9 ± 0.6⁵ |
| PMA, 1 μM | 16.3 ± 1.2⁵ | 15.2 ± 0.9⁵ | 15.8 ± 0.5⁵ |

* p < 0.01.
and Na⁺,K⁺-ATPase isoforms. Because the two amino acids adjacent to Tyr are not positively charged residues, the motif YEL cannot be considered a typical “Tyr-based” consensus sequence. Thus, it is possible that its interaction with AP-2 in intact cells and in response to a physiological agonist (dopamine) may be facilitated by, or involve the presence of, other accessory proteins present in the endocytic machinery. At present it is difficult to establish the AP-2/Na⁺,K⁺-ATPase structural relationship, although some predictions can be made. Whereas the crystal structure of the Na⁺,K⁺-ATPase is still unknown, the structure of skeletal muscle sarcomplasmatic reticulum calcium ATPase (SERCA) α₁-subunit (which appears to share many common structural features with the Na⁺,K⁺-ATPase) has recently been determined (24). The amino acids of SERCA corresponding to the Na⁺,K⁺-ATPase sequence YENLEL are on the exterior of the molecule N-domain, which contains the nucleotide binding site. If the site within the Na⁺,K⁺-ATPase is as exposed as in the SERCA molecule, it may suggests that it is the activation of AP-2 and not the exposure of the “endocytic sequence” that is regulated by the action of dopamine.

Because the decrease in Na⁺,K⁺-ATPase activity elicited by DA is exclusively mediated by internalization of active Na⁺,K⁺-ATPase molecules (9), we further examined whether the absence of Tyr⁵⁸⁷ in the α₁-subunit resulted in its deficient clathrin-dependent endocytosis. Na⁺,K⁺-ATPase α₁-subunit abundance in clathrin vesicles was determined in vesicles prepared from α-WT or α-Y537A cells that were previously treated with DA. Characterization of CCV preparations was performed, and the results were similar to the ones previously described in our laboratory (2). The incubation time chosen (2.5 min) reflects the maximal incorporation of Na⁺,K⁺-ATPase molecules in CCV obtained from renal proximal tubule cells incubated with DA (2). DA treatment significantly increased the Na⁺,K⁺-ATPase abundance in α-WT, whereas it failed to do so in CCV derived from α-Y537A cells (Fig. 3E). Na⁺,K⁺-ATPase immunoreactivity was present in CCV prepared from the α-Y537A mutants despite lacking the ability to bind AP-2. This could represent a population of Na⁺,K⁺-ATPase molecules present in CCV that originate from the recycling pathway (recombining endosomes) in their way to the plasma membrane (25). Because the latter is a process that is mediated by AP-1, its interaction site within the Na⁺,K⁺-ATPase appears not to be shared with the AP-2 binding motif.

In contrast to DA, phorbol esters stimulate Na⁺,K⁺-ATPase activity in OK cells (5, 15), and this effect is mediated by increasing the number of Na⁺,K⁺-ATPase molecules within the plasma membrane (5). Interestingly, a phorbol ester (PMA) also stimulated enzyme activity (nmol of Rb/mg of protein/min) in OK cells bearing the Y537A mutation (α-WT, vehicle: 9.9 ± 0.6 versus 1 μM PMA, 16.3 ± 1.2 and α-Y537A; vehicle: 9.7 ± 0.7 versus 1 μM PMA, 14.0 ± 8.8, n = 3 in all groups), indicating that the stimulatory mechanisms remained intact and that the Y537A mutation appears to affect specifically the inhibitory response to DA. Additionally, these results suggest that the interaction of AP-1 with the Na⁺,K⁺-ATPase molecule that is needed for its recruitment to the plasma membrane in response to phorbol esters (5) requires a different recognition sequence within the α₁-subunit.

Whereas the AP-2 μ-chain interacts with a NPXY or YppØ motif, the β-chain of AP-2 recognizes instead dileucine motifs (–)2–4xLL, where (–) is usually a negatively charged residue and x a polar residue (22). Further analysis of the Na⁺,K⁺-ATPase α₁-subunit sequence revealed the presence of two possible interacting dileucine motifs (EPKHL⁹⁹⁹ and L⁵⁵⁴LLPDE). Na⁺,K⁺-ATPase activity was determined in α-WT cells and in cells stably expressing the α₁-subunit carrying a mutation Leu⁵⁴⁹ → Ala (α-L499A) or Leu⁵⁴⁴ → Ala (α-L554A). The presence of these mutations within the Na⁺,K⁺-ATPase α₁-subunit neither affected the inhibitory action of dopamine nor the stimulatory effect of phorbol esters (Table I). In summary, the present study demonstrates the Y⁵⁸⁷LEL motif as the recognition site within the Na⁺,K⁺-ATPase molecule (catalytic α-subunit) for its interaction with AP-2, a mandatory link in the signaling cascade that translates G protein-coupled receptor activation into clathrin-dependent endocytosis of Na⁺,K⁺-ATPase molecules. Moreover, our data indicate that this motif is not involved in the stimulation/recruitment of Na⁺,K⁺-ATPase molecules (AP-1-dependent) to the plasma membrane induced by phorbol esters and supports the concept that endocytosis and recruitment are two processes involving separate target motifs within the Na⁺,K⁺-ATPase.

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