Distinct Role of the N-terminal Tail of the Na,K-ATPase Catalytic Subunit as a Signal Transducer

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Abbreviations: InsP3, inositol 1,4,5-trisphosphate; InsP3R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; GST, glutathione S-transferase. GSH, Glutathione; RPT, rat proximal tubule.
Mounting evidence suggests that the ion pump, Na,K-ATPase, can, in the presence of ouabain, act as a signal transducer. A prominent binding-motif, linking the Na,K-ATPase to intracellular signaling effectors, has however not yet been identified. Here we report that the N-terminal tail of the Na,K-ATPase catalytic α-subunit (αNT-t) binds directly to the N-terminus of the inositol 1,4,5-trisphosphate receptor (InsP$_3$R). Three amino acid residues, LKK, conserved in most species and most α-isoforms, are essential for the binding to occur. In wild-type cells, low concentrations of ouabain trigger low frequency calcium oscillations that activate NF-κB and protect from apoptosis. All these effects are suppressed in cells over-expressing a peptide corresponding to αNT-t but not in cells over-expressing a peptide corresponding to αNT-tΔLKK. Thus we have identified a well-conserved Na,K-ATPase motif that binds to the InsP$_3$R and can trigger an anti-apoptotic calcium signal.

The Na,K-ATPase is an integral plasma membrane protein that establishes the electrochemical gradient across the plasma membrane in all mammalian cells. Ouabain is a steroid derivative, that binds specifically to Na,K-ATPase. Several recent studies suggest that the ouabain/Na,K-ATPase-complex may act as a signal transducer and transcription activator (1-4) modulating cell growth (5,6), apoptosis (7), and cell motility (8). These effects have been ascribed to the activation of a number of intracellular signaling pathways, for review see (9,10). Most, if not all, of these pathways involve release of calcium (Ca$^{2+}$) from intracellular stores via the inositol 1,4,5-trisphosphate receptor (InsP$_3$R), and results from recent studies indicate that Na,K-ATPase tethers the InsP$_3$R into a Ca$^{2+}$-regulatory complex (3,4). The exact mechanisms by which Na,K-ATPase activates the InsP$_3$R remains to be elucidated. Here we report that the N-terminal tail of the Na,K-ATPase catalytic α-subunit binds to the N-terminus of the InsP$_3$R. Interaction between Na,K-ATPase and InsP$_3$R modulates the Ca$^{2+}$ oscillatory signal which serves to protect the cell from apoptosis (11). The identification of a distinct motif in the Na,K-ATPase, that via protein-protein interaction transmits this signal to the InsP$_3$R have important implications for the many vital cell functions that are regulated by ouabain/Na,K-ATPase-signaling.

**Experimental Procedures**

**Cells and tissue**

Two types of cells were used. COS-7 cells, a cell line derived from fetal monkey kidney, were used in most protocols. Since transformed cells are not suitable for serum deprivation-induced apoptosis, rat proximal tubule (RPT) cells in primary culture were used in these protocols. COS-7 cells were purchased from ECACC (European Collection of Cell Cultures) and were maintained in Dulbecco’s Modified Eagle’s Medium (Sigma), supplemented with 10% fetal bovine serum and 2 mM L-glutamine. RPT cells were prepared as described previously (1). Briefly, kidneys of 20-day-old male Sprague–Dawley rats were used and cells were cultured in supplemented DMEM on glass coverslips for 24 h. Lysates from whole brain and kidney cortex from male adolescent Sprague–Dawley rats were used in co-immunoprecipitation studies.

**Plasmids and transfection**

All plasmids were propagated in *E. coli* strain DH5α. All polymerase chain reaction (PCR) products of cDNA fragments were generated in frame using Platinum® $P_{fx}$ DNA Polymerase (Invitrogen) and were verified by nucleotide sequencing. GFP-Na,K-ATPase α1 and GFP-Na,K-ATPaseα1ΔNT-t were prepared as described previously (3). The Na,K-ATPase α2 and α3 plasmids were kind gifts from Dr. Thomas Pressly, Texas Tech University, and Dr. Jerry Lingrel, University of Cincinnati. PCR products of GFP cDNA fragment was subcloned, into the site of HindIII of the Na,K-ATPase α2 plasmid to
generate GFP-Na,K-ATPase α2 and into the site of HindIII and SacII of the Na,K-ATPase α3 plasmid to generate GFP-Na,K-ATPase α3. The PCR product of mRFP was subcloned into the site of EcoRI and EcoRV of pcDNA4/Myc-His B (Invitrogen) to generate pcDNA4-mRFP (the original mRFP cDNA was a kind gift from Dr. Roger Tsien, University of California, San Diego). Truncated cDNA fragments corresponding to different lengths of the N-terminal fragments of rat Na,K-ATPase α1 were subcloned into the site of HindIII and BamHI of pEGFP-C1 (Clontech) to generate GFP-αNT-t, GFP-αNT-tΔLKK, and into the site of EcoRV and XhoI of pcDNA4-mRFP to generate mRFP-αNT-t and mRFP-αNT-tΔLKK (both of these two plasmids contain a stop codon before the XhoI site). The PCR products corresponding to different lengths of the N-terminal fragments of human Na,K-ATPase α1 or rat Na,K-ATPase α3 were subcloned into the site of HindIII and BamHI of pEGFP-C1 (Clontech) to generate GFP-hαNT-t, GFP-α3NT-t, and GFP-α3NT-tΔLKK.

The PCR products corresponding to the suppressor domain and InsP3-binding domain (a.a. 1-604 of mouse InsP3R type-1) were subcloned into the site of BamHI and EcoRI of pGEX-KG (12) to generate GST-InsP3R(1-604) and into BamHI and XhoI of pET-23a(+) (Novagen) to generate InsP3R(1-604)-His. GST-InsP3R(1-225), GST-InsP3R(1-343) and GST-InsP3R(226-604) were generated as previously described (13).

Transfections were performed using Effectene Transfection Reagent (Qiagen) according to the manufacturer’s protocol. Transfected COS-7 cells were harvested for binding assay or used for Ca2+-imaging experiments 1-2 days after transfection.

**Purification of recombinant proteins and GST pull-down assays**

The plasmids encoding GST- or His-fusion proteins were transformed into *E. coli* BL21 (DE3) pLysS (Stratagene). Harvested *E. coli* expressed with GST fusion proteins or GST alone was sonicated in buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM 2ME, 0.1 % Triton X-100). The lysates of *E. coli* or rat brains were centrifuged at 800 x g for 10 min at 4°C. The supernatants were then subjected to another centrifugation at 20000 x g for 30 min at 4°C and the supernatants from the second centrifugation were used for pull-down binding assay. The strategy for GST pull-down assay was modified from previous description (14). For each reaction, 200 µg protein of solubilized lysates of *E. coli* expressed with GST fusion proteins or GST alone were incubated with 30 µl of 1:1 slurry of Glutathione Sepharose 4B (Amersham Pharmacia Biotech) at 4°C for 1 h and then washed with Buffer C (4 mM Hepes, 100 mM NaCl, 0.1 % TritonX-100) 3 times. The spun down complexes of Glutathione Sepharose 4B, including the fusion proteins, were incubated with 200 µg proteins of solubilized lysates of rat brain for 2 h or over night at 4°C. The complexes were then spun down and washed with Buffer D (Buffer C + 50 mM NaCl) 3 times. The proteins were eluted by boiling in 60 µl of 2 X SDS-PAGE sample buffer for 3 minutes, were separated by SDS-PAGE and then were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were probed with anti-GFP Ab or anti-Na,K-ATPase α1 Ab. For *in vitro* binding assays, GST or GST-αNT-t were eluted with reduced Glutathione (Sigma), InsP3R(1-604)-His was purified with ProBond resin (Invitrogen). All three proteins were dialyzed in Buffer C. 15 µg of dialyzed GST, GST-αNT-t were incubated with 5-15 µg of InsP3R(1-604)-His for 2 h or over night at 4°C. The total volume for each reaction was 1 ml. The complexes were spun down and washed with Buffer D 3 times and then subjected to SDS-PAGE for Western blotting assay or CBB staining.

**Calcium imaging and power spectrum analysis**

COS-7 cells were loaded with 5 µM Fura2/AM (Molecular Probes) at room temperature for 1 h. Calcium measurements were performed at ~37°C in a heated chamber (QE-1, Warner Instruments) with a cooled
CCD camera (ORCA-ERG, Hamamatsu) mounted on an upright microscope (Axioskop 2 FS, Zeiss) with a 40x 0.8 NA water dipping lens. Excitation at 340 and 380 nm was performed with a monochromator (Polychrome IV, TILL Photonics). All devices were controlled and data was analyzed with computer software (MetaFluor, Molecular Devices). Sampling frequencies were between 0.05 and 0.1 Hz. All experiments were performed in physiological buffer (100 mM NaCl, 4 mM KCl, 20 mM HEPES, 25 mM NaHCO3, 1.5 mM CaCl2, 1.1 mM MgCl2, 1 mM NaH2PO4, 10 mM D-Glucose, pH 7.4).

To determine the number of oscillating cells, an oscillating cell was defined as a cell that displayed at least two well defined Ca2+ peaks, where each peak value was an increase in Ca2+ of more than 10% compared to the baseline. Spectral analysis of Ca2+ oscillations was performed with MATLAB software as described previously (15). The red fluorescent protein mRFP was used, since its spectral properties are better in combination with Fura2/AM compared to GFP. The Ca2+-imaging studies were performed by an investigator blind to the transfection protocol.

Detection and quantification of apoptotic cells (TUNNEL staining)

We have previously found that ouabain protects from serum deprivation-induced apoptosis, but not from apoptosis triggered by 0.5 µM staurosporin (11). Since cell lines are resistant to serum deprivation-induced apoptosis, we used RPT cells in primary culture in this protocol. This cell type has previously been shown to respond to ouabain with Ca2+ oscillations and downstream NF-κB activation (1,3). Cells were cultured with 10% FBS or 0.2% FBS for 24 h and treated with 10 nM ouabain. To obtain a semi-quantitative estimation of the level of apoptosis the TUNNEL assay (ApopTag Red In Situ Apoptosis Detection kit, Chemicon Int., USA) was carried out according to the manufacturer’s instructions. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI 1.5 µg/ml).

Cells were examined in a Leica TCS SP inverted confocal scanning laser microscope using a 40x/1.4 N.A. oil-immersion objective. ApopTag Red fluorescence was excited at 543 nm and detected with a 560-620 nm band pass filter. DAPI stained cells were viewed with UV light. Apoptotic index (AI), i.e. number of apoptotic cells/total number of cells counted×100, was determined by counting the number of ApopTag positive cells over total number of cells, determined by DAPI staining. In each preparation 8-10 randomly selected areas were examined, and in each area 100-200 DAPI stained cells were counted. Approximately 1000 DAPI stained cells were counted in each preparation.

NF-κB activity assay

NF-κB translocation to nucleus was used as an index of NF-κB activation and studied with immunocytochemistry. Immunocytochemistry was performed as previously described (1).

Rubidium uptake

The dose-dependent effect of ouabain on Na,K-ATPase activity was determined by measuring ouabain-sensitive 86Rb+ uptake as described previously (16).

Antibodies and chemicals

The following antibodies and chemicals were purchased: mouse anti-GFP monoclonal antibody (Clontech), anti-Na,K-ATPase α1 monoclonal antibody (Upstate Biotechnology), mouse anti-His monoclonal antibody (Invitrogen), Rabbit anti-NF-κB polyclonal antibody (Santa Cruz Biotechnology), ouabain and bradykinin (Sigma), Fura2/AM (Invitrogen), thapsigargin (Calbiochem).

Statistics

Data are presented as mean ± SEM. Student’s t-test and one-way ANOVA with Bonferroni post hoc test was used and significance was accepted at p < 0.05.

Results

Na,K-ATPase α1, α2, and α3-subunits interact with the N-terminus of the InsP₃R

The InsP₃R is a protein of 2749 residues. There are three functionally distinct regions within the InsP₃R, the long N-terminal portion of the receptor, the channel-forming region, and the short C-terminal regulatory segment (Fig. 1A). To screen for an interaction
between the Na,K-ATPase and the InsP$_3$R, we first incubated Glutathione S-transferase (GST) fused proteins encoding for various lengths of the N-terminus of the InsP$_3$R type 1 with lysates from whole rat brain or with lysates from COS-7 cells transfected with GFP-fused rat Na,K-ATPase $\alpha$-subunits. Consistent interaction was found between the Na,K-ATPase $\alpha_1$-subunit and peptide fragments corresponding to residues 1-604 of InsP$_3$R type 1. This fragment of InsP$_3$R type 1 consists of a suppressor domain and an InsP$_3$-binding domain (17) and shares high homology with other subtypes of InsP$_3$R. Since the main goal of this study was to identify an InsP$_3$R binding site in the Na,K-ATPase molecule, all subsequent studies were performed with peptide fragments corresponding to various lengths of the 1-604 portion of the InsP$_3$R type 1.

As shown in Fig. 1, GST fusion proteins encoding for various lengths of InsP$_3$R(1-604) were incubated with lysates of whole rat brain and rat kidney cortex and pulled-down with Glutathione (GSH)-Sepharose (Fig. 1B). GST-InsP$_3$R(1-604) and GST-InsP$_3$R(1-343), which encodes the suppressor domain and the first 118 residues of the InsP$_3$-binding domain, which encodes only the suppressor domain, and GST-InsP$_3$R(226-604), which encodes only the InsP$_3$-binding domain, did not (Fig. 1B). To examine whether InsP$_3$R(1-604) also assembles with other Na,K-ATPase $\alpha$-isoforms, the GST fusion proteins were incubated with lysates of COS-7 cells transfected with GFP-fused rat Na,K-ATPase $\alpha_1$, $\alpha_2$, and $\alpha_3$. As shown in Fig. 1C, GST-InsP$_3$R(1-343) and GST-InsP$_3$R(1-604) were found to assemble with GST-Na,K-ATPase $\alpha_1$, $\alpha_2$ and $\alpha_3$. GST-InsP$_3$R(1-225), GST-InsP$_3$R(226-604) and GST alone did not assemble with any of the $\alpha$-isoforms.

The N-terminal tail of Na,K-ATPase $\alpha$-subunit is responsible for binding to InsP$_3$R(1-604)

Since it was suggested from previous studies that the N-terminal tail of the Na,K-ATPase $\alpha$-subunit plays an important role for Na,K-ATPase signaling (3,4), we examined whether GST-InsP$_3$R(1-604) would assemble with a truncated form of the Na,K-ATPase $\alpha$-subunit, where the first 32 residues of the N-terminal tail had been deleted (GFP-Na,K-ATPase$_{\alpha\Delta NT-t}$) and/or with a peptide fragment, corresponding to the N-terminal tail of Na,K-ATPase $\alpha_1$ (GFP-$\alpha$NT-t) (Fig. 2(A,B)). These constructs were expressed in COS-7 cells and pull-down assays were performed. As shown in Fig. 2C, GST-InsP$_3$R(1-604) assembled with GFP-$\alpha$NT-t. In contrast, GST-InsP$_3$R(1-604) did not assemble with GFP-Na,K-ATPase$_{\alpha\Delta NT-t}$.

The well-conserved amino acid residues LKK in the N-terminal tail of Na,K-ATPase $\alpha$-subunit are essential for the interaction with InsP$_3$R

Since the studies shown in Fig. 1C demonstrated that GST-InsP$_3$R(1-604) also assembled with the Na,K-ATPase $\alpha_2$- and $\alpha_3$-isoforms, we next examined the homology of the N-terminal tail. The N-terminal tail of different isoforms and species displayed little homology, except for 3 amino acid residues, LKK, which are conserved in all Na,K-ATPase $\alpha$-subunit isoforms examined (Fig. 3A).

To study the role of the LKK residues for the Na,K-ATPase-InsP$_3$R interaction, a peptide-fragment corresponding to the N-terminal tail of the rat Na,K-ATPase $\alpha_1$ subunit, in which the LKK residues were deleted, was generated (GFP-$\alpha$NT-t$_\Delta$LKK). This fragment did not assemble with GST-InsP$_3$R(1-604) (Fig. 3(B,C)). In contrast, a peptide fragment corresponding to the human $\alpha_1$ N-terminal tail, which has little homology with the rat $\alpha_1$ N-terminal tail, except for the LKK motif, did assemble with GST-InsP$_3$R(1-604) (Fig. 3(D,E)). To further examine the role of the conserved LKK for the interaction, two different lengths of the N-terminal tail of rat Na,K-ATPase $\alpha_3$-subunit, with or without the conserved LKK, were constructed and used for pull-down assay. As shown in Fig. 3(F,G), the fragment of the N-terminal tail of rat Na,K-ATPase $\alpha_3$-subunit lacking the conserved LKK did not assemble with GST-InsP$_3$R(1-604), whereas the fragment of the N-terminal tail of rat Na,K-ATPase $\alpha_3$-subunit with the conserved LKK, did assemble with GST-InsP$_3$R(1-604).
To determine whether the Na,K-ATPase α-subunit can bind directly to the InsP₃R(1-604), InsP₃R(1-604) was tagged with His (InsP₃R(1-604)-His) (Fig. 4A) and expressed in E. coli. Purified InsP₃R(1-604)-His was incubated with purified GST-αNT-t and then precipitated with GSH-Sepharose. As depicted in Fig. 4(B,C), GST-αNT-t did bind to InsP₃R(1-604)-His.

The first 5 residues, which are not present in the mature Na,K-ATPase α₁-subunit protein, may block Na,K-ATPase-InsP₃R interaction

The first 5 residues of rat Na,K-ATPase α₁-subunit, MGKGV, are considered to be absent in the mature protein (18). The physiological significance of these 5 residues is unknown. To examine whether these 5 residues have an effect on Na,K-ATPase-InsP₃R interaction, GFP-MGKGV-αNT-t were expressed in COS-7 cells and pull-down assays were performed. The GFP fusion prevents the cleavage of MGKGV (18). As shown in Fig. 5(A,B), GFP-MGKGV-αNT-t did not assemble with GST-InsP₃R(1-604), suggesting that these 5 residues of the Na,K-ATPase α₁-subunit may inhibit the Na,K-ATPase-InsP₃R interaction. To further test this possibility, we recombined these first 5 residues of the α₁-subunit with the N-terminal tail of α₃-subunit. GFP-MGKGV-α₃NT-t expressed in COS-7 cells was subjected to GST-InsP₃R(1-604) for pull-down assay. As shown in Fig. 5(C,D), GFP-MGKGV-α₃NT-t did not assemble with GST-InsP₃R(1-604). These findings indicate that these 5 amino acid residues of the Na,K-ATPase block the Na,K-ATPase-InsP₃R interaction and interfere with the signaling function of Na,K-ATPase α₁.

Role of the Na,K-ATPase N-terminus for ouabain-induced Ca²⁺ oscillations

Next we examined the impact of the Na,K-ATPase N-terminus for the functional interaction between Na,K-ATPase and the InsP₃R. We have previously shown that ouabain enhances the interaction between Na,K-ATPase and the InsP₃R and triggers an oscillatory Ca²⁺ response that is dependent on release of Ca²⁺ via the InsP₃R. Those studies were performed mainly on rat renal cells in primary culture. A similar oscillatory response to ouabain is also observed in COS-7 cells (Fig. 6(A,B)). The majority of ouabain-exposed cells displayed regular Ca²⁺ oscillations with an initial stable baseline. The Ca²⁺ oscillations were analyzed using power spectral analysis and revealed an oscillatory period of 3.9 ± 0.2 min for 0.2 µM ouabain (66 cells from 7 experiments) and 3.3 ± 0.1 min for 1 µM ouabain (95 cells from 9 exp) treatment (Fig. 6(C,D)). The number of cells responding with Ca²⁺ oscillations increased dose-dependently with increasing ouabain concentration (Fig. 6E, open circles). The threshold concentration of ouabain required for triggering Ca²⁺ oscillations gave less than 10 % inhibition of Rb⁺ uptake, used as an index of Na,K-ATPase dependent ion flux (Fig. 6E, filled circles). The off-rate for ouabain to Na,K-ATPase is much slower than the on-rate (19), and if cells were exposed to 10-50 nM ouabain for several hours, Ca²⁺ oscillations were observed in 5-30 % of COS-7 cells. Ouabain failed to induce Ca²⁺ oscillations in cells that had been treated with thapsigargin to deplete the endoplasmic stores of Ca²⁺ (Fig. 6F). The ouabain response was then studied in cells transfected with truncated form of the α-subunit, where the first 32 amino acid residues were deleted. The truncated α-subunit was fused to GFP. The number of GFP positive cells responding with Ca²⁺ oscillations was significantly lower than the number of GFP negative cells responding to ouabain (44.8 ± 7.9 % and 70.1 ± 6.7 % respectively) (Fig. 6G). GFP positive and GFP negative cells on the same plate were compared. Cells expressing GFP alone showed a similar ouabain-response as GFP negative cells (data not shown).

To study whether overexpression of αNT-t would result in a constitutive activation of InsP₃R, leading to lower Ca²⁺ levels in ER, the Ca²⁺ content in ER was evaluated by estimating the release of Ca²⁺ from the ER following inhibition of the SERCA pump by thapsigargin. As shown in Fig. 7A, the Ca²⁺ release from the ER was similar in cells over-
expressing mRFP-αNT-t and mRFP-αNT-tΔLKK.

Thereafter we examined whether the αNT-t peptide might act as an inhibitor of ouabain-induced Ca\(^{2+}\) oscillations. Overexpression of the fusion peptide mRFP-αNT-t resulted in a robust decline of the number of cells responding to 0.2 μM ouabain (37.3 ± 8.0 % for mRFP positive cells and 63.2 ± 4.7 % for mRFP negative cells) (Fig. 7B). In contrast, the number of ouabain responding cells was unaltered following overexpression of mRFP-αNT-tΔLKK (73.5 ± 7.9 % for mRFP positive cells and 73.6 ± 4.8 % for mRFP negative cells).

To test whether the αNT-t peptide might act as an inhibitor of InsP\(_3\) activation of the InsP\(_3\)R Ca\(^{2+}\) release channel, cells were exposed to bradykinin to stimulate the generation of endogenous InsP\(_3\). The number of responding cells (Fig. 7C) as well as the amplitude and full with half maximum (data not shown) was similar in wild-type cells and cells over-expressing mRFP-αNT-t and mRFP-αNT-tΔLKK.

**Ouabain protection from serum deprivation-induced apoptosis and activation of NF-κB is mediated via the Na,K-ATPase α1-subunit N-terminal tail**

Slow Ca\(^{2+}\) oscillations have been shown to activate the Ca\(^{2+}\)-dependent transcription factor NF-κB (20). NF-κB has in many systems an anti-apoptotic effect (21,22), and we have, in a recently completed study, shown that non-inhibitory doses of ouabain protect from serum deprivation-induced apoptosis (11). Here we have examined whether expression of the αNT-t may attenuate the ouabain-induced protection from apoptosis. Since cell lines are fairly independent of growth factors, we used RPT cells in primary culture to provoke serum deprivation-induced apoptosis. This cell type has previously been shown to respond to low doses of ouabain with Ca\(^{2+}\) oscillations (3). RPT cells expressing GFP-αNT-t, GFP-αNT-tΔLKK or GFP only, were incubated with 10 nM ouabain for 24 h. NF-κB activation was estimated by measuring the ratio of NF-κB nuclear to cytosolic signal. In cells expressing GFP only or GFP-αNT-tΔLKK, exposure to ouabain caused a significant increase in NF-κB nuclear to cytosolic ratio (154.8 ± 3.0 % and 129.3 ± 7.3 % respectively). In contrast, the ratio was not affected by ouabain in cells expressing GFP-αNT-t (93.0 ± 4.1 %).

To explore the ouabain-induced protection from apoptosis the apoptotic index (AI) was measured. In each protocol AI was measured from more than 1000 cells. During control conditions AI ranges between 1 and 4 %. In serum deprived cells, AI ranged between 10 and 15 %. Ouabain failed to protect from serum deprivation-induced apoptosis in RPT cells expressing GFP-αNT-t, but not in cells expressing GFP-αNT-tΔLKK (Fig. 7D). Representative confocal images of RPT cells treated with 10 nM ouabain and transfected with either GFP-αNT-tΔLKK or GFP-αNT-t and stained using TUNNEL assay for apoptosis are shown in Fig. 7(E-J). RPT cells were grown in 0.2 % FBS for 24 h in the presence or absence of 10 nM ouabain.

**Discussion**

Na,K-ATPase is a well-studied molecule in its role as the main determinant of the sodium/potassium gradient across the plasma membrane in most eukaryotic cells (23-25). The minimal functional unit is a heterodimer of a catalytic α-subunit and a β-subunit that is required for proper Na,K-ATPase plasma membrane insertion (26). Recent work from many laboratories has demonstrated that Na,K-ATPase is an important signaling receptor. Ouabain, a steroid hormone generally considered to be produced in the adrenals and hypothalamus (27), is a highly specific ligand of the Na,K-ATPase α-subunit. Many recent studies have indicated that ouabain-bound Na,K-ATPase can, independent of its ion transporting function, activate a number of signaling pathways (9,10) and modulate cell growth (5,6), migration (8) and programmed cell death (7). Low doses of ouabain have in several cell types been shown to activate InsP\(_3\)R and trigger intracellular Ca\(^{2+}\) oscillation (1,3). Recent work from many laboratories has begun to define the Na,K-ATPase signalosome and to map the
functional domains that are involved in the organization of the individual signaling. The present study provides the first documentation that Na,K-ATPase can regulate cellular functions via direct interaction with an intracellular signaling molecule. We show here that the N-terminal tail of the Na,K-ATPase α-subunit binds to InsP₃R and that a peptide fragment, corresponding to the N-terminal tail of Na,K-ATPase α-subunit, will attenuate ouabain-induced Ca²⁺ oscillations as well as the down-stream effects on NF-κB activation and apoptosis. The Na,K-ATPase α-subunit can tolerate extensive mutation within the N-terminal tail without compromising the pump function, and a number of studies have shown the first 32 amino acid residues of the rat Na,K-ATPase catalytic α₁-subunit, the α₁ isoform most extensively used in this study, can be deleted without changes in overall Na,K-ATPase function (28-30). Thus the Ca²⁺ signaling function of Na,K-ATPase resides in a segment of the molecule that is not essential for pump function.

The mammalian catalytic subunit of Na,K-ATPase exists in at least 4 isoforms (31). The α₁-isoform is ubiquitously expressed, while the other isoforms, such as the neuron-specific α₃-isoform, are more tissue specific (32). All isoforms of rat Na,K-ATPase α-subunit interacted with the InsP₃R. Furthermore the N-terminal tail of both human and rat α₁- and α₃-subunit were found to interact with InsP₃R(1-604) (Fig. 3). Although all Na,K-ATPase α isoforms share more than 80 % homology, the N-terminal tail shows little homology between the different isoforms (33). One exception is the lysine-rich motif (LKK) in the N-terminal tail that is conserved in almost all species. This well-conserved motif was found to be essential for the binding between the Na,K-ATPase α-subunit and the InsP₃R, indicating the universal importance of this protein–protein interaction.

The N-terminus of the InsP₃R consists of two distinct functional units, the suppressor domain (a.a. 1-225) and the InsP₃-binding domain (a.a. 226-579). Na,K-ATPase α₁-subunit did not interact with either functional unit alone, but did interact with the suppressor domain and the first 118 amino acids of the InsP₃-binding domain (InsP₃R(1-343)). The crystal structure of the suppressor domain of the InsP₃R and the InsP₃-binding domain have recently been solved (17,34). As discussed by Bosanac et al. (34), a highly conserved surface on the InsP₃-binding domain, including amino acids E283, V286, K306 and Y313, is a likely site for interaction with other proteins, such as the Na,K-ATPase α-subunit. Functional data from this and previous study imply that the binding sites for the αNT-t and the binding site for the InsP₃ molecule do not overlap. Bradykinin, a well-known trigger of intracellular InsP₃ generation, produced the same Ca²⁺ response in cells over-expressing the αNT-t as in wild-type cells. Ouabain-induced Ca²⁺ oscillations are still observed in the presence of PLC inhibitor as well as in cells expressing a peptide that binds InsP₃ with much higher affinity than the InsP₃R (3).

The exact mechanism by which the ouabain/Na,K-ATPase complex triggers repetitive release of Ca²⁺ from the InsP₃R channel remains to be determined, but we speculate that the αNT-t acts as an allosteric modulator of the InsP₃R, altering its channel properties. Based on the knowledge of the crystal structure of the SERCA pump, the cytoplasmic domains of the Na,K-ATPase α-subunit has been organized into three domains, the nucleotide binding domain, the phosphorylation domain and the actuator domain (A domain) (35). The A domain contains the N-terminus and is involved in the transmission of conformational changes. Ouabain shifts the conformational equilibrium of Na,K-ATPase, and this should affect the position of the A domain. Since the N-terminus of the A domain is associated with the InsP₃R, it is likely that this will also lead to an allosteric modification of the InsP₃R molecule.

Release of Ca²⁺ from the intracellular stores via the InsP₃R is the major determinant of ouabain-induced Ca²⁺ oscillations (3). The InsP₃R is a modulator of a wide variety of functions, such as cell proliferation, differentiation, apoptosis, fertilization, behavior and memory (36,37). Activation of the InsP₃R can result in single or repeated transient increases in intracellular Ca²⁺.
periodicity of the repeated, oscillatory, Ca\(^{2+}\) response can, depending on the activator, vary from seconds to hours. This temporal diversity is of utmost importance for the versatility of the Ca\(^{2+}\) signaling, since most cells have tools to encode the frequency of Ca\(^{2+}\) oscillations (36). Ouabain-induces Ca\(^{2+}\) oscillations in COS-7 cells revealed a periodicity of approximately 4 min while in RPT cells the periodicity was approximately 5 min (1). Calcium oscillations with a periodicity in this range will typically activate the transcription factor NF-\(\kappa\)B (20), which in many systems has an anti-apoptotic effect (21,22).

We have in a recently completed study demonstrated that ouabain fails to protect from serum deprivation-induced apoptosis in cells where the ER stores have been Ca\(^{2+}\) depleted (11). We also demonstrated that long term exposure to low dose of ouabain, 10 nM for 24 h, triggered a nuclear translocation of NF-\(\kappa\)B and that protection from apoptosis was abolished in cells exposed to an inhibitor of NF-\(\kappa\)B. These results were confirmed in the present study. Over-expression of \(\alpha\)NT-t significantly blocked the effects of ouabain on intracellular Ca\(^{2+}\) oscillation and NF-\(\kappa\)B activation. Ouabain-dependent protection from apoptosis was completely abolished in cells over-expressing \(\alpha\)NT-t. These data indicate that the \(\alpha\)NT-t peptide acts as a dominant negative inhibitor of the down-stream effects of the Na,K-ATPase-InsP\(_3\)R interaction.

A comparison between the effect of ouabain-bound Na,K-ATPase and the effect of the pro-apoptotic protein cytochrome C, which binds to the C-terminus of the InsP\(_3\)R, on Ca\(^{2+}\) release from the InsP\(_3\)R, illustrates the versatility of InsP\(_3\)R dependent Ca\(^{2+}\) signaling. The periodicity of ouabain/Na,K-ATPase triggered oscillations is lower than the cytochrome C triggered oscillations, which are in the range of 20 min (38). Thus a Ca\(^{2+}\) oscillatory signal, triggered by the direct interaction between the InsP\(_3\)R and another protein, may be pro-apoptotic or anti-apoptotic depending on the periodicity of the resulting Ca\(^{2+}\) oscillation. Given this intriguing role of the InsP\(_3\)R as one of the modulators of the threshold for apoptosis, the question whether Na,K-ATPase binding to InsP\(_3\)R suppresses the apoptotic effects of cytochrome C interaction with InsP\(_3\)R will be an interesting possibility for future studies.

References


Footnotes

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The authors declare that no conflict of interest exists.
Figure Legends

Fig. 1. Binding of InsP₃R(1-604) to mammalian Na,K-ATPase α-subunits. A. Schematic representation of mouse InsP₃R type-1 and the peptide fragments corresponding to various lengths of the N-terminus of the InsP₃R fused with GST. B. The GST-fusion proteins or GST alone attached to GSH-Sepharose were incubated with the lysates of whole rat brain or rat kidney cortex and subjected to Western blotting with anti-Na,K-ATPase α₁ antibody. GST-InsP₃R(1-343) and GST-InsP₃R(1-604) bind to Na,K-ATPase α₁, whereas GST-InsP₃R(1-225), GST-InsP₃R(226-604) and GST does not. C. The GST-fusion proteins or GST alone attached to GSH-Sepharose were incubated with the lysates of COS-7 cells expressing GFP-Na,K-ATPase α₁, GFP-Na,K-ATPase α₂ or GFP-Na,K-ATPase α₃. The proteins were subjected to Western blotting with anti-GFP antibody. GST-InsP₃R(1-343) and GST-InsP₃R(1-604) bind to GFP-Na,K-ATPase α₁, GFP-Na,K-ATPase α₂ and GFP-Na,K-ATPase α₃, whereas GST-InsP₃R(1-225), GST-InsP₃R(226-604) and GST do not.

Fig. 2. The N-terminal tail of Na,K-ATPase α-subunits is necessary and sufficient for binding to InsP₃R(1-604). A. Schematic structure of the rat Na,K-ATPase α₁ and the amino acids sequence of the N-terminal cytoplasmic tail. B-C. GST-InsP₃R(1-604) attached to GSH-Sepharose was incubated with the lysates of COS-7 cells expressing GFP-Na,K-ATPase α₁, GFP-Na,K-ATPase α₂ or GFP-Na,K-ATPase α₃. The proteins were subjected to Western blotting with anti-GFP antibody. GST-InsP₃R(1-343) and GST-InsP₃R(1-604) bind to GFP-Na,K-ATPase α₁, GFP-Na,K-ATPase α₂ and GFP-Na,K-ATPase α₃, whereas GST-InsP₃R(1-225), GST-InsP₃R(226-604) and GST do not.

Fig. 3. The three residues, LKK, conserved in the Na,K-ATPase α-subunit of all species, are essential for the interaction with the InsP₃R(1-604). A. Sequence alignment of the N-terminal tails of Na,K-ATPase α-subunits. Conserved residues are highlighted in grey. B-C. GST-InsP₃R(1-604) attached to GSH-Sepharose was incubated with the lysates of COS-7 cells expressing GFP-αNT-t or GFP-αNT-tΔLKK (B). The proteins were subjected to Western blotting with anti-GFP antibody (C). GFP-αNT-t binds to GST-InsP₃R(1-604), whereas GFP-αNT-tΔLKK does not. D-E. GST-InsP₃R(1-604) or GST attached to GSH-Sepharose were incubated with the lysate of COS-7 cells expressing GFP-hαNT (D). The proteins were subjected to Western blotting with anti-GFP antibody (E). GFP-hαNT-t binds to GST-InsP₃R(1-604), but not to GST. F-G. GST-InsP₃R(1-604) attached to GSH-Sepharose was incubated with the lysates of COS-7 cells expressing GFP-α₃NT-t or GFP-α₃NT-tΔLKK (F). The proteins were subjected to Western blotting with anti-GFP antibody (G). GFP-α₃NT-t binds to GST-InsP₃R(1-604), whereas GFP-α₃NT-tΔLKK does not.

Fig. 4. Na,K-ATPase α-subunits bind directly to the InsP₃R(1-604). Purified InsP₃R(1-604)-His was incubated with purified GST-αNT-t (A) or GST and then precipitated with GSH-Sepharose. The proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) (B) or probed with anti-His antibody (C). GST-αNT-t binds to InsP₃R(1-604)-His, whereas GST does not. The arrows indicate the size for InsP₃R(1-604)-His.

Fig. 5. The first 5 residues, which are generally considered to be cleaved in the mature Na,K-ATPase α₁-subunit, can block the Na,K-ATPase-InsP₃R interaction. A-B. GST-InsP₃R(1-604) attached to GSH-Sepharose was incubated with the lysates of COS-7 cells expressing GFP-MGKGV-αNT-t or GFP-αNT-t (A). The proteins were subjected to Western blotting with anti-GFP antibody (B). GFP-αNT-t binds to GST-InsP₃R(1-604), but GFP-MGKGV-αNT-t does not. C-D. GST-InsP₃R(1-604) attached to GSH-Sepharose was incubated with the lysates of COS-7
cells expressing GFP-MGKGV-α3NT-t or GFP-α3NT-t (C). The proteins were subjected to Western blotting with anti-GFP antibody (D). GFP-α3NT-t binds to GST-InsP₃R(1-604), whereas GFP-MGKGV-α3NT-t does not.

**Fig. 6.** Ouabain-induced Ca²⁺ oscillations in COS-7 cells. **A-B.** Slow regular Ca²⁺ oscillations were observed in cells loaded with a Ca²⁺ sensitive dye (Fura2/AM) and treated with 0.2 μM or 1.0 μM ouabain. [Ca²⁺]i (a.u.) represents ratio of the Fura2/AM images (340/380 nm), corresponding to changes in intracellular Ca²⁺ levels. **C-D.** Power spectral analysis of the two ouabain-evoked Ca²⁺ oscillations depicted in A and B respectively. For each concentration the mean period (T ± SEM) of the oscillations was calculated from more than 65 cells from at least 7 different experiments. **E.** Dose dependent effect of ouabain. Filled circles show percent inhibition of Rb⁻-uptake, used as an index of K⁺ uptake for different ouabain concentrations. Open circles show percent oscillating cells in response to different ouabain concentrations. **F.** Ouabain does not trigger Ca²⁺ oscillations following depletion of intracellular Ca²⁺ stores with thapsigargin. **G.** Over-expression of a truncated Na,K-ATPase α₁, where 32 amino acids from the N-terminus were deleted (GFP-Na,K-ATPaseα1ΔNT-t), significantly suppressed the ouabain-induced Ca²⁺ oscillations.

**Fig. 7.** Over-expression of αNT-t attenuates ouabain-induced Ca²⁺ oscillations, NF-κB activation, and the anti-apoptotic effect. **A.** Ca²⁺ release from the endoplasmic stores in response to thapsigargin was similar in COS-7 cells over-expressing mRFP-αNT-t and mRFP-αNT-tΔLKK. **B.** Over-expression of mRFP-αNT-t resulted in inhibition of the ouabain-induced Ca²⁺ oscillations in COS-7 cells, whereas over-expression of mRFP-αNT-tΔLKK had no effect. **C.** Percentage cells responding to bradykinin was similar in COS-7 cells over-expressing mRFP-αNT-t and mRFP-αNT-tΔLKK. **D-J.** Ouabain failed to protect from serum deprivation-induced apoptosis in RPT cells expressing GFP-αNT-t, but not in cells expressing GFP-αNT-tΔLKK (D). Representative confocal images of RPT cells treated with 10 nM ouabain and transfected with GFP-αNT-tΔLKK (E) or GFP-αNT-t (H) and stained using TUNNEL assay for apoptosis (F and I) and merged images (G and J).
Fig. 1

A

<table>
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<tr>
<th>Suppressor domain</th>
<th>InsP₃-binding domain</th>
<th>Modulatory &amp; transducing domain</th>
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<td>2276</td>
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GST-InsP₃R(1-225)

aa. 1

225

GST-InsP₃R(1-343)

aa. 1

343

GST-InsP₃R(1-604)

aa. 1

604

GST-InsP₃R(226-604)

aa. 226

604

B

Marker (kDa)

Input

GST

GST-InsP₃R(1-225)

GST-InsP₃R(1-343)

GST-InsP₃R(1-604)

GST-InsP₃R(226-604)

100

Brain lysate

150

WB: Anti-Na,K-ATPaseα1 Ab

100

Kidney cortex lysate

C

Marker (kDa)

Input

GST

GST-InsP₃R(1-225)

GST-InsP₃R(1-343)

GST-InsP₃R(1-604)

GST-InsP₃R(226-604)

100

GFP-Na,K-ATPaseα1

150

GFP-Na,K-ATPaseα2

100

GFP-Na,K-ATPaseα3

WB: Anti-GFP Ab
Fig. 2

A

Extracellular

PM

Cytoplasmic

NH₂

COOH

(MGKGV) GRDKYEPAVSEHGDKKSKKAKKERMDDELKK

B

GFP-Na,KATPaseα1

GFP

aa.6

1023

GFP-Na,KATPaseα1ΔNT-t

GFP

aa.37

1023

GFP-αNT-t

GFP

aa.6

37
**Fig. 3**

**A**

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<tr>
<td>C</td>
<td></td>
<td></td>
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<tr>
<td>WB: anti-GFP Ab</td>
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<td>Pellet</td>
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</table>

**B**

GFP-αNT-t | GFP | aa.6 | 37 |

GFP-αNT-tΔLKK | GFP | aa.6 | 34 |

**C**

Input | GST | GST-InsP(1-604) | rat4 | human Na,K-ATPaseα1 |

**D**

human Na,K-ATPaseα1 | GFP-αNT-t | GFP | aa.6 | 37 |

**E**

Input | GST | GST-InsP(1-604) | rat4 | human Na,K-ATPaseα1 |

**F**

rat Na,K-ATPaseα3 | GFP-α3NT-t | GFP | aa.1 | 27 |

GFP-α3NT-tΔLKK | GFP | aa.1 | 24 |
**Fig. 4**

A

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B

![CBB Staining](image)

C

![WB: anti-His (C) Ab](image)
Fig. 5

A

GFP-MGKGV-αNT-t

MGKGV

aa.1 5 37

GFP

GFP-αNT-t

GFP

aa.6 37

B

Marker (kDa)

Input

37

25

WB: anti-GFP Ab

Pellet

C

GFP-MGKGV-α3NT-t

MGKGV

aa.1 5 aa.1 27

GFP

α1

α3

GFP-α3NT-t

GFP

α3

aa.1 27

D

Input

37

25

WB: anti-GFP Ab

Pellet

25
Fig. 6

A. 0.2 μM Ouabain

B. 1.0 μM Ouabain

C. T = 3.9 ± 0.2 min

D. T = 3.3 ± 0.1 min

E. Frequency (mHz)

F. Power (a.u.)

G. Frequency (mHz)

H. Power (a.u.)

I. Frequency (mHz)

J. Power (a.u.)

K. Frequency (mHz)

L. Power (a.u.)

M. Frequency (mHz)

N. Power (a.u.)

O. Frequency (mHz)

P. Power (a.u.)

Q. Frequency (mHz)

R. Power (a.u.)

S. Frequency (mHz)

T. Power (a.u.)

U. Frequency (mHz)

V. Power (a.u.)

W. Frequency (mHz)

X. Power (a.u.)

Y. Frequency (mHz)

Z. Power (a.u.)

[Ca^{2+}]_i (a.u.)

Time (min)

Frequency (mHz)

Power (a.u.)

Ouabain (μM)

% Oscillating cells

Na,K-ATPase α1 ΔNT-t

Control cells

Thapsigargin

Ouabain

[Ca^{2+}]_i (a.u.)

Time (min)

Frequency (mHz)

Power (a.u.)

Ouabain (μM)

% Oscillating cells

Na,K-ATPase α1 ΔNT-t

Control cells

Thapsigargin

Ouabain

[Ca^{2+}]_i (a.u.)

Time (min)

Frequency (mHz)

Power (a.u.)

Ouabain (μM)

% Oscillating cells

Na,K-ATPase α1 ΔNT-t

Control cells

Thapsigargin

Ouabain

[Ca^{2+}]_i (a.u.)

Time (min)

Frequency (mHz)

Power (a.u.)

Ouabain (μM)

% Oscillating cells

Na,K-ATPase α1 ΔNT-t

Control cells

Thapsigargin

Ouabain
Fig. 7

A

[Ca^{2+}]_i (a.u.)

0 100 200 300 400

time (s)

Thapsigargin

αNT-t

αNT-tΔLKK

B

Oscillating cells (%)

100

50

0

αNT-t

αNT-tΔLKK

control cells

Ouabain

C

Responding cells (%)

100

50

0

αNT-t

αNT-tΔLKK

control cells

Bradykinin

D

Apoptotic Index (%)

14

7

0

αNT-t

αNT-tΔLKK

ouabain

Apoptosis

E

GFP signal

GFP-αNT-tΔLKK

F

Apoptag Red signal

G

merge

H

GFP signal

GFP-αNT-t

I

Apoptag Red signal

J

merge
Table 1. Description of the plasmids used in this study

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Distinct role of the N-terminal tail of the Na,K-ATPase catalytic subunit as a signal transducer

Songbai Zhang, Seth Malmersjo, Juan Li, Hideaki Ando, Oleg Aizman, Per Uhlen, Katsuhiko Mikoshiba and Anita Aperia

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