Ankyrin B Modulates the Function of Na,K-ATPase/Inositol 1,4,5-Trisphosphate Receptor Signaling Microdomain*

Xiao Liu‡1, Zuzana Špicarová, Susanna Rydholm†, Juan Li‡, Hjalmar Brismar§, and Anita Aperia‡2

From the ‡Department of Woman and Child Health, Karolinska Institutet, Astrid Lindgren Children’s Hospital, Q2:09, SE-171 76 Stockholm, Sweden and §Department of Cell Physics, Royal Institute of Technology, AlbaNova University Center, 106 91 Stockholm, Sweden

Na,K-ATPase and inositol 1,4,5-trisphosphate (IP3) receptor (IP3R) can form a signaling microdomain that in the presence of ouabain triggers highly regular calcium oscillations. Downstream effects include NF-κB activation. Here we report that ankyrin B (Ank-B), expressed in most mammalian cells, plays a pivotal role in the function of the Na,K-ATPase/IP3R signaling microdomain. In studies performed on a monkey kidney cell line, we show that Ank-B co-precipitates with both Na,K-ATPase and IP3R. We identify the N terminus tail of the Na,K-ATPase catalytic subunit and the N-terminal portion 1–604 of the IP3R as novel binding sites for Ank-B. Knockdown of Ank-B with small interfering RNA reduced the expression of Ank-B to 15–30%. This down-regulation of Ank-B attenuated the interaction between Na,K-ATPase and IP3R, reduced the number of cells responding to ouabain with calcium oscillations, altered the calcium oscillatory pattern, and abolished the ouabain effect on NF-κB. In contrast, Ank-B down-regulation had no effect on the ion transporting function of Na,K-ATPase and no effect on the distribution and apparent mobility of Na,K-ATPase in the plasma membrane.

Na,K-ATPase is an energy transducing ion-pump expressed in all mammalian cells. Recent studies have revealed a new role for Na,K-ATPase as a signal transducer. Ouabain, an analogue of digoxin and a highly specific ligand of Na,K-ATPase, has been shown by several groups to trigger intracellular signaling pathways controlling cell proliferation, apoptosis, cell motility, heart contractility, and blood pressure (1–3). Results from our laboratory have demonstrated that Na,K-ATPase and the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R) can form a signaling microdomain that, in the presence of ouabain, triggers slow calcium oscillations and activates the NF-κB signaling protein may support the interaction between Na,K-ATPase and IP3R and, if so, what the functional role of this protein can be.

Ankyrins belong to a ubiquitously expressed intracellular scaffolding protein family that includes Ank-B, Ank-G, and Ank-R (7). Ankyrins associate with a diverse set of membrane, cytoskeletal, and cytoplasmic proteins and tether them into specialized membrane signaling domains. Both Ank-B and Ank-G have been reported to interact with Na,K-ATPase, but Ank-B is the only ankyrin isoform that has been reported to interact with IP3R (8–10). Thus, Ank-B is a likely protein to be a partner of an Na,K-ATPase/IP3R signaling microdomain. Previous studies performed with a pulldown technique have suggested that ankyrin may bind to the second cytoplasmic subunit of Na,K-ATPase and the C terminus of the IP3R (11, 12). Here we have examined whether Ank-B may bind directly to the N-terminal tail of the catalytic α-subunit of Na,K-ATPase and the N-terminal portion 1–604 of the IP3R, i.e. the sites for Na,K-ATPase/IP3R interaction. We found this to be the case.

To test the functional role of Ank-B in ouabain/Na,K-ATPase/IP3R signaling, a cell line derived from fetal monkey kidneys, COS-7, was used. This cell line has previously been shown to respond to ouabain with slow calcium oscillations (4, 6). With use of small interfering RNA (siRNA) we specifically knocked down Ank-B and compared the calcium and NF-κB response to ouabain in wild type and Ank-B-silenced cells. Cells transfected with control siRNA were used as an additional control. The signaling response to ouabain was found to be altered in Ank-B-silenced cells. Determination of the plasma membrane expression of Na,K-ATPase and of its ion transporting function indicated that the effect of Ank-B silencing on Na,K-ATPase signaling was not due to internalization of the protein.

MATERIALS AND METHODS

Cell Culture and Transfection—COS-7 cells, a cell line derived from fetal monkey (Cercopithecus aethiops) kidney (ECACC no. 87021302) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mm l-glutamine. Plasmid DNA and synthetic siRNA duplexes were transfected using either Effectene or HiPerFect according to the manufacturer’s protocol (Qiagen). siRNA optimal concentra-
Role of Ank-B in Na,K-ATPase/IP3R Signaling

tion was determined by titration to 10 nM. siRNAs were fluorescence-tagged with Label IT® CyTM3 labeling kit (Mirus) according to the manufacturer’s protocol. Transfected cells were used for experiments 2 days after transfection unless otherwise stated.

Measurement of Cell Viability and Proliferation—A WST-1 assay (Chemicon International) was used to measure cell viability in Ank-B siRNA and rat siRNA-transfected cells as described previously (3). Bromodeoxyuridine incorporation was used to determine cells proliferation. The cell proliferation kit (GE Healthcare) was used according to manufacturer’s protocol.

DNA Plasmid Constructs and siRNA—A full-length human Na,K-ATPase α1 subunit (hNa,K-ATPase α1) was constructed and cloned into pEGFP-C2 vector. This construct was modified to obtain cDNA that encodes hNa,K-ATPase α1 with truncation of first five N-terminal residues (MGKGV), which are cleaved during the posttranslational modification of the enzyme (14). The glutathione S-transferase (GST)-fused N-terminal fragment of Na,K-ATPase α1 (6–100 aa) and the fragment corresponding to the 1–604 residues of IP3R type 1 were generated as described previously (4, 6). The monkey ankyrin B-targeted sequence is based on the sequence of Ank-B from *Macaca mulatta*, the closest available monkey Ank-B sequence in the data base (XM_001094169): AAC CTG GAC AAA GTC GTG GAA. For controls, we used siRNA generated by changing three nucleotides (bold) from targeted sequence AAC CTG to TAG GTA CAG GTT GAA or siRNA based on rat Ank-B sequence (XM_227735): CGG GCA CAC TGT GGT GAA. RNA oligonucleotides were synthesized by Qiagen.

RNA Isolation and Reverse Transcription-PCR—Total RNA was extracted from siRNA-transfected cells using Aurum Total RNA mini kit (Bio-Rad) according to manufacturer’s protocol.

First-strand cDNA was synthesized from 2 µg of total RNA using Moloney murine leukemia virus reverse transcriptase and oligonucleotide (dT) primers (Promega). PCR primers were designed according to the sequence from monkey Ank-B (XM_001094169) (forward, 5'–CAC AAG GCT CCT GTT GTG GAA or siRNA based on rat Ank-B sequence (XM_227735): CGG GCA CAC TGT GGT GAA) and PCR was carried out with standard conditions.

Purification of Recombinant GST-fused Proteins and in Vitro Binding Analysis—The GST-fused fragment of N-terminal Na,K-ATPase α1, GST-fused fragment corresponding to residues 1–604 of IP3R, and GST alone were produced in *Escherichia coli* BL21. Bacteria were sonicated in the buffer A (10 mM Heps (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM 2-β-mercaptoethanol, 0.5% Triton X-100, and protease inhibitor mixture (Roche Diagnostics)). The lysate was centrifuged at 12,000 × g for 30 min at 4 °C. Supernatant was incubated with glutathione-Sepharose 4B beads (GE Healthcare) for 1 h at 4 °C, and the beads were washed with buffer A + 200 mM NaCl 4 times.

30 µl of 50% slurry Sepharose beads with bound purified GST-fused proteins or GST alone were incubated with 400 ng of purified human Ank-B (a generous gift from Dr. Vann Bennett) in buffer B (20 mM Heps (pH 7.4), 40 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor mixture (Roche Diagnostics)) for 1 h at 4 °C. The beads were then washed with buffer B + 1 mM NaCl four times. Proteins were eluted with 25 µl of 2X Laemmli buffer for 5 min at 100 °C. Samples were subjected to SDS-PAGE for Western blotting or Coomassie Brilliant Blue staining.

Antibodies and Chemicals—For immunoprecipitation, the following antibodies were used: mouse monoclonal anti-Na,K-ATPase α1 (Upstate Biotechnology, clone 6H), mouse monoclonal anti IP3R3 (BD Transduction Laboratories; clone 2), mouse monoclonal anti-Ank-B (Zymed Laboratories Inc.; clone 2.2), and control mouse IgG (Sigma-Aldrich). For Western blot analysis the following antibodies were used: mouse monoclonal anti Na,K-ATPase α1 (Upstate Biotechnology; clone 6H), rabbit polyclonal Pan IP3Rs (Chemicon), mouse monoclonal anti-Ank-B (a gift from Dr. Vann Bennett, Duke University), mouse monoclonal anti-actin antibody (BD Transduction Laboratories, clone C4), and secondary antibodies against rabbit or mouse IgG conjugated with horseradish peroxidase (GE Healthcare). All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

Immunoprecipitation—Cells were incubated with 20 pm ouabain for 30 min, washed 2 times with phosphate-buffered saline (PBS), lysed on ice in cold buffer (50 mM Tris/HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5% sodium deoxycholate, 1.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche Diagnostics)), sonicated, and centrifuged at 25,000 × g for 60 min at 4 °C. Supernatant protein concentration was measured by Bio-Rad DC protein assay (Bio-Rad) using bovine serum albumin as the standard.

Protein solutions were normalized to an equivalent amount of total protein. The protein solution (800 µg) was incubated with 3 µg of anti-Na,K-ATPase α1 antibody, anti-IP3R3 antibody, anti-Ank-B antibody, or IgG for 2 h at 4 °C. Immunocomplexes were then incubated with 30 µl of 50% slurry of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) overnight at 4 °C with end-to-end mixing. The next day the beads were washed 4 times with lysis buffer, and proteins were eluted with 30 µl of 2X Laemmli sample buffer and heated at 37 °C for 15 min.

Western Blotting—Proteins solubilized in Laemmli sample buffer were resolved in 7 or 12% polyacrylamide gels by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were then blocked in 5% nonfat milk in TBS-T (136 mM NaCl, 10 mM Tris, 0.05% Tween 20) and immunoblotted using the corresponding primary antibodies for 1 h at room temperature. After 3 washes with TBS-T, the membranes were incubated with secondary antibodies labeled with horseradish peroxidase (GE Healthcare) for 1 h at room temperature. Membranes were washed 3 times with TBS-T, and the protein bands were visualized by chemiluminescence using chemiluminescent substrate ECL (GE Healthcare).

Cell Surface Biotinylation—Cells cultured on 60-mm dishes were exposed to EZ-link Sulfo-NHS-SS-biotin (Pierce) at a final concentration 1.5 mg/ml in PBS at 4 °C for 60 min with gentle shaking. After washing twice with PBS and once with PBS containing 100 mM glycine to quench unreacted biotin, the cells were lysed in ice-cold radioimmune precipitation assay buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 1% Nonidet P-40,
cells were pretreated with ouabain (20 μM) for 20 min at 37 °C.

Ratiometric Imaging of Intracellular Calcium—COS-7 cells were loaded with the calcium-sensitive dye Fura-2/AM (Molecular Probes) (5 μM in PBS) for 1 h at room temperature. Loaded cells were pretreated with ouabain (20 μM) for 20 min at 37 °C. Ratiometric imaging of intracellular calcium was performed in the presence of ouabain using a heated Petri dish holder mounted on a Zeiss Axioskop 2 microscope with a 40×/0.8 dipping objective. Loaded cells were excited at wavelength 340 and 380 nm, and emission fluorescence was detected with a CCD camera (Hamamatsu ORCA-ER C4742–95) via an image intensifier unit (Hamamatsu C9016). Ratio images were recorded every 8–20 s for 45 min. One dish was used for each experiment. On each dish 15–20 cells, generally within the same cluster, were analyzed. Oscillating cells were defined as cells that display at least two well-defined Ca²⁺ peaks. Spectral analysis of Ca²⁺ oscillations was performed using MATLAB (6).

Fluorescence Recovery after Photobleaching (FRAP)—FRAP is a well established method used to study the mobility of proteins (16). Experiments were carried out using a Zeiss 510LSM laser scanning confocal microscope equipped with a 63×/0.9 dipping objective. A 488-nm laser line was used for both bleaching and imaging. COS-7 cells were transfected with siRNA 1 day after plating and with plasmid coding for green fluorescent protein (GFP) fused to Na,K-ATPase (GFP-Na,K-ATPase) the following day. The cells were used for experiments 2 days after the last transfection. Bleaching of a plasma membrane section was performed with 100% laser power for 10 s, and fluorescence recovery images were recorded with 3% laser power every 5 s for 10 min. Images were analyzed by using Image Examiner (Carl Zeiss, Jena, Germany) and ImageJ (NIH). On each cell the same size and shape of area was bleached. This area was then used for calculation of average fluorescence intensity. Data were corrected for bleaching and normalized against the initial intensity.

NF-κB Activity—NF-κB translocation to nucleus was used as an index of NF-κB activation. Non-transfected wild type (WT), rat siRNA, and Ank-B siRNA-transfected COS-7 cells were treated with 10 μM ouabain for 24 h. The subcellular localization of NF-κB was determined using TransAM™ NF-κB p65 kit (Active Motif) following the manufacturer’s protocol. Nuclear extracts were harvested from cells as described previ-
ty-eight hours after siRNA transfection the expression of Ank-B mRNA (data not shown) and protein were reduced. The protein level in Ank-B siRNA-transfected cells ranged between 15 and 30% that detected in mismatch and rat siRNA-transfected and non-transfected wild type cells (Fig. 4). The Ank-B silencing had no effect on cell viability, cell proliferation, or morphology (data not shown). Notably, Na,K-ATPase α1-IP3R interaction was dramatically diminished in Ank-B-silenced cells but not in the mismatch and rat siRNA-transfected cells (Fig. 5). Because similar negative results were obtained with rat and mismatch siRNA, we used rat siRNA as the control siRNA in the subsequent experiments.

Ouabain binding to Na,K-ATPase triggers highly regular calcium oscillations in COS-7 cells as well as in several other cell types. The association between Na,K-ATPase and IP3R is a prerequisite for this effect to occur. Partial disruption of the Na,K-ATPase/IP3R interaction with an inhibitory peptide was found to reduce the number of cells responding with calcium oscillations (6). To examine the functional role of Ank-B in the interaction between Na,K-ATPase and IP3R, we compared the calcium response to ouabain in wild type, control siRNA, and monkey Ank-B siRNA-transfected cells. We used a concentration of 20 pM ouabain. This concentration does not give any detectable inhibition of pump activity and is in the range of the physiological ouabain concentration (17). The number of responding cells was significantly lower in monkey Ank-B siRNA-transfected cells than in wild type cells and rat siRNA-transfected cells (Fig. 6A). The calcium spike frequency was significantly different in cells where Ank-B had been down-regulated (4.9 ± 1.5 mHz) as compared with wild type and rat siRNA-transfected cells (7 ± 1.5 and 6.7 ± 1.3 mHz, respectively).

Ank-B Silencing Abolishes NF-κB Activation by Ouabain—NF-κB activation is an important downstream effect of the ouabain/Na,K-ATPase/IP3R signaling pathway (3, 5, 6). To test whether Ank-B can modulate the NF-κB response to ouabain, control and Ank-B-silenced cells were exposed to a non-inhibitory dose of ouabain or to a vehicle for 24 h. The translocation of NF-κB from the cytoplasm to the nucleus was studied either by subcellular fractionation and detection of activated NF-κB or with immunofluorescent labeling of NF-κB in fixed cells. In both non-transfected wild type cells and control siRNA-transfected cells we observed a significant translocation of NF-κB to the nucleus after ouabain treatment. In contrast, no changes in
the subcellular distribution of NF-κB in Ank-B-silenced cells were observed (Fig. 7, A and B).

Ank-B Silencing Does Not Affect the Ion-transporting Capacity and Plasma Membrane Distribution of Na,K-ATPase—To test whether silencing of Ank-B has an effect on the ion transporting function of Na,K-ATPase, we compared ouabain-sensitive Rb⁺ uptake in Ank-B-silenced cells and in control cells. Ouabain-sensitive Rb⁺ uptake was similar in both groups (Table 1).

To determine the surface distribution of the Na,K-ATPase, a surface biotinylation assay was performed. The fraction of the Na,K-ATPase α1 that was distributed in the plasma membrane in Ank-B-silenced cells and in control siRNA-transfected cells was similar (Fig. 8, A and B).

Plasma Membrane Na,K-ATPase Mobility in Control and Ank-B-silenced Cells—To test whether the interaction between Na,K-ATPase and Ank-B has an effect on the mobility of Na,K-ATPase within the plasma membrane, we measured the FRAP of green fluorescent protein-labeled Na,K-ATPase α1 subunit in COS-7 cells. On each experimental day cells transfected with control, and Ank-B siRNA were studied in parallel. The recovery pattern of Na,K-ATPase is seen in Fig. 9A, and recovery curves showing a characteristic membrane diffusion behavior are shown in Fig. 9B. The diffusion rate was similar for control siRNA-transfected and Ank-B-silenced cells. From the recovery curves the mobile pool (i.e. the diffusible fraction of Na,K-ATPase molecules) can be calculated (16, 18). The mobile pool was defined as the percentage of recovery reached 6 min after bleaching. In control siRNA-transfected cells the mobile pool of Na,K-ATPase averaged 65 ± 3%. In Ank-B-silenced cells the mobile pool was similar, 66 ± 2%. Fig. 9C shows values of Na,K-ATPase mobile pool measured for each single cell.

DISCUSSION

We have previously demonstrated the existence of a signaling microdomain that contains Na,K-ATPase and IP3R and
induces Ca\(^{2+}\)/H11001 oscillations and activation of NF-\(\kappa\)B. Here we show that Ank-B is an active partner of this signaling microdomain. We show that Ank-B serves to stabilize the interaction between Na,K-ATPase and IP3R and that Ank-B is required for the stabilization of the signaling function of the Na,K-ATPase/IP3R complex. Calcium signaling from the Na,K-ATPase/IP3R complex is triggered by the interaction between the N terminus of Na,K-ATPase \(\alpha1\) subunit and the N-terminal portion 1–604 of the IP3R (6). Results from the present study show that these regions of Na,K-ATPase \(\alpha1\) and IP3R molecules bind directly to Ank-B. These two N termini represent novel binding sites for Ank-B. Zhang et al. (19) reported in 1998 that ankyrin binds to aa 142–166 in the second cytoplasmic domain of Na,K-ATPase. Bourguignon and Jin (13) reported in 1995 that ankyrin binds to aa 2548–2558 in the C terminus of the IP3R. This motif is located very close to the IP3R channel structure. The interaction between the N terminus of the IP3R and ankyrin was not studied. The N terminus of the IP3R consists of two distinct functional units, the suppressor domain (aa 1–225) and the IP3 binding domain (aa 226–579). The crystal structure of these domains has recently been solved (20). A highly conserved surface on the IP3 binding domain was found to be a particularly suitable site for interaction with other proteins. The binding of Ank-B to the N termini of Na,K-ATPase \(\alpha1\) and IP3R plays an important role in bringing Na,K-ATPase \(\alpha1\) and IP3R together. In Ank-B-silenced cells, the interaction between Na,K-ATPase \(\alpha1\) and IP3R was reduced as indicated by the co-immunoprecipitation studies.

**TABLE 1**

| Na,K-ATPase activity in control siRNA and monkey Ank-B-silenced cells (Ank-B siRNA) | Ouabain-sensitive Rb uptake* |
|---|---|
| Control siRNA | 69.6 ± 4.2 |
| Ank-B siRNA | 62.1 ± 2.2 |

* \(^{86}\)Rb uptake (ps/min/mg) of total protein.

**FIGURE 7.** Ank-B is necessary for ouabain-activated NF-\(\kappa\)B activation in COS-7 cells. A, the chemiluminescence intensity signal, which represents the chemiluminescence intensity signal, which represents activated Na,K-ATPase, translocated to the nucleus, in WT, control siRNA, and monkey Ank-B siRNA-transfected COS-7 cells after exposure to 10 ps ouabain for 24 h. The signal from ouabain-treated cells is given in percentage of the signal from control cells, studied in parallel. The experiment was repeated three times. B, representative confocal images of Na,K-ATPase and NF-\(\kappa\)B immunofluorescence signal in non-transfected (WT) and monkey Ank-B siRNA-transfected COS-7 cells in the absence or presence of 10 ps ouabain.

**FIGURE 8.** Examination of Na,K-ATPase surface expression using membrane-impermeable biotinylation reagent. A, the control siRNA and monkey Ank-B siRNA-transfected COS-7 cells were biotinylated according to the description under “Material and Methods.” The same volume of the unbound fraction (U) and total cell lysate (T) was subjected to SDS-PAGE and probed with Na,K-ATPase antibody. B, the data from three experiments were quantified. The fraction of unbound (U) Na,K-ATPase is shown as a percentage of the total cell lysate (T).

**FIGURE 9.** Ank-B down-regulation has no effect on Na,K-ATPase mobile pool in the plasma membrane. A, images from different time points of a FRAP experiment. B, example curves from FRAP experiment. The curves have not reached steady state after 380 s due to continuous exchange of Na,K-ATPase-proteins in the membrane. a.u., arbitrary units. C, percentage of Na,K-ATPase (NKA) mobile pool in control siRNA and monkey Ank-B siRNA-transfected cells. Each cross corresponds to a single cell; lines represent the median.

**TABLE 1**

| Na,K-ATPase activity in control siRNA and monkey Ank-B-silenced cells (Ank-B siRNA) | Ouabain-sensitive Rb uptake* |
|---|---|
| Control siRNA | 69.6 ± 4.2 |
| Ank-B siRNA | 62.1 ± 2.2 |

* \(^{86}\)Rb uptake (ps/min/mg) of total protein.

**FIGURE 7.** Ank-B is necessary for ouabain-activated NF-\(\kappa\)B activation in COS-7 cells. A, the chemiluminescence intensity signal, which represents activated Na,K-ATPase, translocated to the nucleus, in WT, control siRNA, and monkey Ank-B siRNA-transfected COS-7 cells after exposure to 10 ps ouabain for 24 h. The signal from ouabain-treated cells is given in percentage of the signal from control cells, studied in parallel. The experiment was repeated three times. B, representative confocal images of Na,K-ATPase and NF-\(\kappa\)B immunofluorescence signal in non-transfected (WT) and monkey Ank-B siRNA-transfected COS-7 cells in the absence or presence of 10 ps ouabain.

**TABLE 1**

| Na,K-ATPase activity in control siRNA and monkey Ank-B-silenced cells (Ank-B siRNA) | Ouabain-sensitive Rb uptake* |
|---|---|
| Control siRNA | 69.6 ± 4.2 |
| Ank-B siRNA | 62.1 ± 2.2 |

* \(^{86}\)Rb uptake (ps/min/mg) of total protein.
The studies of the calcium and NF-κB responses to ouabain indicate that Ank-B association with both Na,K-ATPase and IP3R has implications for Na,K-ATPase/IP3R signaling. Calcium is a promiscuous messenger that can interact with numerous downstream effectors. The calcium signals vary in their spatial and temporal dynamics (21). Oscillations reduce the threshold for activation of calcium-dependent transcription factors and provide by their level of frequency specificity to the response. Several types of non-excitable cells, including renal proximal tubule cells, astrocytes, and COS-7 cells, respond to ouabain with slow calcium oscillation (4, 5, 9). Power spectrum analysis generally reveals a highly regular oscillatory pattern. Release of calcium from intracellular stores plays a key role for the generation of calcium oscillations (22). Ouabain-activated calcium release from intracellular stores is due to interaction between IP3R with the N terminus of Na,K-ATPase and is independent of IP3 (4). Results from the present study indicate that Ank-B plays a modulatory role in mediating the ouabain-triggered oscillatory response. Using a physiological dose of ouabain, we observed a robust decrease in the number of responding cells in the Ank-B-silenced group as compared with the control group. Qualitative differences in the response were also observed.

Calcium oscillations may be the most versatile of all cell signals, since the cell can decode the frequency of the oscillations (21). The transcription factor NF-κB is typically activated by low frequency calcium oscillations (23). Ouabain-triggered calcium oscillations will activate NF-κB in a variety of cell types (3, 5, 9). Ouabain did not activate NF-κB in the Ank-B-silenced cells but did so in cells transfected with control Ank-B siRNA. This finding emphasizes the importance of the calcium oscillatory pattern for the cellular read-out. NF-κB is a pleiotropic transcriptional factor. In most cell systems activation of NF-κB has an anti-apoptotic effect (24, 25). We have recently shown that exposure to low doses of ouabain can protect renal epithelial cells exposed to serum starvation-triggered apoptosis (3). The present results imply that Ank-B may play an active role for this anti-apoptotic effect to occur.

Our results suggest that although Ank-B is important for the interaction between Na,K-ATPase and IP3R, it may play a minor role in the targeting of Na,K-ATPase to the plasma membrane. We found no indication of internalization of Na,K-ATPase in the Ank-B-silenced cells, and according to the rubidium uptake studies, there was little effect of Ank-B silencing on the Na,K-ATPase pumping capacity. In Ank-B−/− cardiomyocytes, the relative fraction of Na,K-ATPase in the plasma membrane was decreased by 16%, as judged from ouabain binding studies (26). The Ank-B-silenced cells expressed ∼20% of the normal level Ank-B. This difference is likely attributed to different cell types.

Several intracellular signaling pathways may be triggered by the ouabain/Na,K-ATPase complex. In a series of studies, Xie and co-workers (27–29) have demonstrated that Na,K-ATPase associates with Src and that ouabain binding to Na,K-ATPase leads to Src release from Na,K-ATPase/Src complex. One of many effects of ouabain-mediated release and subsequent activation of Src is tyrosine phosphorylation of the IP3R and release of Ca2+ from the intracellular stores (28). The role of Ank-B for the association between Na,K-ATPase and Src remains to be determined. Recently the group of Xie (30) presented evidence suggesting the existence of two pools of Na,K-ATPase in the plasma membrane, a pumping and a non-pumping pool. Furthermore, their results suggested that the non-pumping pool may, at least in some types of cells, function as the ouabain binding signaling transducer. In the FRAP studies we found that the mobile pool of Na,K-ATPase was 65%, and the immobile pool was 35%. Silencing of Ank-B had no measurable effect on Na,K-ATPase mobility. Our results from the FRAP studies would be compatible with the model proposed by Xie, assuming that 1) it is mainly the non-pumping pool of Na,K-ATPase that associates with Ank-B and 2) it is the caveolae structures that stabilize and anchor non-pumping Na,K-ATPase in the plasma membrane. The role of Ank-B may then be to stabilize the signaling complex of Na,K-ATPase and IP3R.

Studies on Ank-B-deficient mice have shown that many important cellular processes are Ank-B-dependent. Ankyrin-B−/− mice display bradycardia, variable heart rate, and stress and exercise-induced polymorphic ventricular arrhythmia and death. Humans with mutations in the Ank-B gene display a spectrum of cardiac arrhythmias (26, 31). These defects have been attributed to loss of Ank-B tethering of a number of ion transporters, including Na,K-ATPase. Because loss of Ank-B in cardiomyocytes affects the excitability of these cells, it is possible that the pathophysiology may be attributed to dysfunction of Na,K-ATPase ion transport rather than to dysfunction of Na,K-ATPase signaling. Ank-B−/− mice die at postnatal days 1–2 (32) and display defects in the nervous system including hypoplasia of the corpus callosum and pyramidal tracts, dilation of lateral ventricles, and degeneration of long axon tracts (33). These phenotypes indicate a critical role for ankyrin B in brain development. The results from the present study have revealed a novel role for Ank-B as a modulator of the ouabain/Na,K-ATPase signaling pathways. It will be an important topic for future studies to explore to what extent these malformations in the brain and other organs may be due to loss of Na,K-ATPase/IP3R signaling.

Acknowledgments—We thank Eivor Zettergren Markus and Markus Krausmann for experimental assistance and Thomas Liebmann for careful reading of the manuscript.

REFERENCES

1. Schoner, W., and Scheiner-Bobis, G. (2007) An. J. Physiol. Cell Physiol. 293, 509–536
2. Xie, Z., and Cai, T. (2003) Mol. Interact. 3, 157–168
3. Li, J., Zelenin, S., Aperia, A., and Aizman, O. (2006) J. Am. Soc. Nephrol. 17, 1848–1857
4. Miyakawa-Naito, A., Uhlen, P., Lal, M., Aizman, O., Mikoshina, K., Brisman, H., Zelenin, S., and Aperia, A. (2003) J. Biol. Chem. 278, 50355–50361
5. Aizman, O., Uhlen, P., Lal, M., Brisman, H., and Aperia, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13420–13424
6. Zhang, S., Malmersjo, S., Li, J., Ando, H., Aizman, O., Uhlen, P., Mikoshina, K., and Aperia, A. (2006) J. Biol. Chem. 281, 21954–21962
7. Mohler, P. J., Gramolini, A. O., and Bennett, V. (2002) J. Cell. Sci. 115, 1565–1566
8. Lencesova, L., O’Neill, A., Resneck, W. G., Bloch, R. J., and Blaustein, M. P. (2004) J. Biol. Chem. 279, 2885–2893
9. Liu, X. L., Miyakawa, A., Aperia, A., and Krieger, P. (2007) Neureport 18,
Role of Ank-B in Na,K-ATPase/IP3R Signaling

597–600

10. Mohler, P. J., Davis, J. Q., and Bennett, V. (2005) PLoS Biol. 3, e423

11. Woroniecki, R., Ferdinand, J. R., Morrow, J. S., and Devarajan, P. (2003) Am. J. Physiol. Renal Physiol. 284, 358–364

12. Devarajan, P., Scaramuzzino, D. A., and Morrow, J. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2965–2969

13. Bourguignon, L. Y., and Jin, H. (1995) J. Biol. Chem. 270, 7257–7260

14. Pressley, T. A., Allen, J. C., Clarke, C. H., Odebunmi, T., and Higham, S. C. (1996) Am. J. Physiol. 271, C825–C832

15. Cheng, X. J., Fisone, G., Aizman, O., Aizman, R., Levenson, R., Greengard, P., and Aperia, A. (1997) Am. J. Physiol. 273, C893–C901

16. Scott, L., Zelenin, S., Malmersjo, S., Kowalewski, J. M., Markus, E. Z., Nairn, A. C., Greengard, P., Brismar, H., and Aperia, A. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 762–767

17. Schoner, W., and Scheiner-Bobis, G. (2007) Am. J. Cardiovasc. Drugs 7, 173–189

18. Choquet, D., and Triller, A. (2003) Nat. Rev. Neurosci. 4, 251–265

19. Zhang, Z., Devarajan, P., Dorfman, A. L., and Morrow, J. S. (1998) J. Biol. Chem. 273, 18681–18684

20. Bosanac, I., Yamazaki, H., Matsu-Ura, T., Michikawa, T., Mikoshiba, K., and Ikura, M. (2005) Mol. Cell 17, 193–203

21. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Nat. Rev. Mol. Cell Biol. 4, 517–529

22. Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S., and Mikoshiba, K. (1992) Science 257, 251–255

23. Dolmetsch, R. E., Xu, K., and Lewis, R. S. (1998) Nature 392, 933–936

24. Mogi, M., Ozeki, N., Nakamura, H., and Togari, A. (2004) Bone (NY) 35, 507–516

25. Nakanishi, C., and Toi, M. (2005) Nat. Rev. Cancer 5, 297–309

26. Mohler, P. J., Schott, J. J., Gramolini, A. O., Dilly, K. W., Guatimosim, S., duBell, W. H., Song, L. S., Haurogne, K., Kyndt, F., Ali, M. E., Rogers, T. B., Lederer, W. J., Escande, D., Le Marec, H., and Bennett, V. (2003) Nature 421, 634–639

27. Tian, J., Cai, T., Yuan, Z., Wang, H., Liu, L., Haas, M., Maksimova, E., Huang, X. Y., and Xie, Z. J. (2006) Mol. Biol. Cell 17, 317–326

28. Yuan, Z., Cai, T., Tian, J., Ivanov, A. V., Giovannucci, D. R., and Xie, Z. (2005) Mol. Biol. Cell 16, 4034–4045

29. Haas, M., Askari, A., and Xie, Z. (2000) J. Biol. Chem. 275, 27832–27837

30. Liang, M., Tian, J., Liu, L., Pierre, S., Liu, J., Shapiro, J., and Xie, Z. J. (2007) J. Biol. Chem. 282, 10585–10593

31. Mohler, P. J., Splawski, I., Napolitano, C., Bottelli, G., Sharpe, L., Timothy, K., Priori, S. G., Keating, M. T., and Bennett, V. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9137–9142

32. Tuvia, S., Buhusi, M., Davis, L., Reedy, M., and Bennett, V. (1999) J. Cell Biol. 147, 995–1008

33. Scotland, P., Zhou, D., Benveniste, H., and Bennett, V. (1998) J. Cell Biol. 143, 1305–1315