Cell Signaling Microdomain with Na,K-ATPase and Inositol 1,4,5-Trisphosphate Receptor Generates Calcium Oscillations

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Running Title: Na,K-ATPase and InsP₃ Receptor in a Signaling Microdomain

1 The abbreviations used are: Ca²⁺, calcium; Na⁺, sodium; K⁺, potassium; InsP₃, inositol 1,4,5-trisphosphate; InsP₃R, inositol 1,4,5-trisphosphate receptor; FRET, fluorescent resonance energy transfer; RPT, renal proximal tubule; ER, endoplasmic reticulum; PLC, phospholipase C; SERCA, sarco-endoplasmic reticulum Ca²⁺ ATPase; CPA, cyclopiazonic acid; 2-APB, 2-aminoethoxydiphenyl borate; SOC, store operated calcium; CytD, cytochalasin D.
Summary

Recent studies indicate novel roles for the ubiquitous ion pump, Na,K-ATPase, in addition to its function as a key regulator of intracellular sodium and potassium concentration. We have previously demonstrated that ouabain, the endogenous ligand of Na,K-ATPase, can trigger intracellular calcium (Ca\(^{2+}\)) oscillations, a versatile intracellular signal controlling a diverse range of cellular processes. Here we report that Na,K-ATPase and inositol 1,4,5-trisphosphate (InsP\(_3\)) receptor (InsP\(_3\)R) form a cell signaling microdomain that, in the presence of ouabain, generates slow Ca\(^{2+}\) oscillations in renal cells. Using fluorescent resonance energy transfer (FRET) measurements, we detected a close spatial proximity between Na,K-ATPase and InsP\(_3\)R. Ouabain significantly enhanced FRET between Na,K-ATPase and InsP\(_3\)R. The FRET effect and ouabain-induced Ca\(^{2+}\) oscillations were not observed following disruption of the actin cytoskeleton. Partial truncation of the NH\(_2\)-terminus of Na,K-ATPase catalytic α1-subunit abolished Ca\(^{2+}\) oscillations and downstream activation of NF-κB. Ouabain-induced Ca\(^{2+}\) oscillations occurred in cells expressing an InsP\(_3\)-sponge and were hence independent of InsP\(_3\) generation. Thus, we present a novel principle for a cell signaling microdomain where an ion pump serves as a receptor.
**Introduction**

Na,K-ATPase is an integral membrane protein expressed in all eukaryotic cells where it functions as a key regulator of intracellular sodium (Na\(^+\))\(^1\) and potassium (K\(^+\)) concentrations (1). Recent studies however, point to an additional role for Na,K-ATPase as a signal transducer (2-5). Importantly, Na,K-ATPase has an endogenous ligand, ouabain, a steroid hormone that dose-dependently inhibits the activity of Na,K-ATPase. The biological role of ouabain is, despite extensive research, not well understood. Ouabain belongs to the family of cardiac glycosides which have been used for centuries in the treatment of heart disease. Recently, several investigators have noted that cardiac glycosides may act as anticancer agents (6,7).

We have described a new cell signaling pathway triggered by ouabain (2). Using rat renal proximal tubule (RPT) cells, we showed that exposure to concentrations of ouabain that cause only partial inhibition of Na,K-ATPase activity induces slow intracellular calcium (Ca\(^{2+}\)) oscillations and subsequent activation of the transcription factors NF-κB and CREB. Our results from that study indicated that Ca\(^{2+}\) oscillations occurred as an interplay between different Ca\(^{2+}\) transporters and that Ca\(^{2+}\) release via the inositol 1,4,5-trisphosphate (InsP\(_3\)) receptor (InsP\(_3\)R) was involved in this event. Na,K-ATPase does not possess the characteristics of a G-protein coupled receptor. Given the generality of Na,K-ATPase expression and its significant role in cell homeostasis, it is important to identify the molecular mechanisms by which Na,K-ATPase can act as a signal transducer. Here we show that the generation of Ca\(^{2+}\) oscillations by ouabain is dependent on the physical association of Na,K-ATPase and InsP\(_3\)R in a signaling microdomain.
Experimental Procedures

Expression Plasmids

A cDNA fragment encoding wild type rat Na,K-ATPase α1-subunit (NKAα1) was amplified by AmpliTaq Gold (Applied Biosystems). The PCR product was digested by Apal/XbaI restriction enzymes and cloned into pEGFP-C2 (Clontech) to obtain pGFP-NKAα1. A mutant NKAα1 with truncation of the first 32 amino acids (NKAα1.M32) was generated using PCR. The region of truncation of the NH2-terminus was decided on the basis of structure/function analysis reported elsewhere (8). The sense primer sequence was 5’-AAAGGGCCCATGGAAGTGTCTATGGACGAC-3’, corresponding to nucleotide positions 349-366 of NKAα1 (RefSeq accession number NM_012504) with additional Apal site and ATG codon on 5’-end of primer. The antisense primer was 5’-CTTGCCGTGGAGGAGGATAGAACT-3’ corresponding to nucleotide positions 1792–1815 of NKAα1. The PCR product and pGFP-NKAα1 were hydrolyzed by Apal/AflII restriction enzymes and ligated for cloning pGFP-NKAα1.M32.

A fusion protein with NH2-terminal glutathione S-transferase (GST) and 95 amino acids of NKAα1 (GST-NKAα1.N95), was constructed using Gateway Technology (Invitrogen). Briefly, a cDNA fragment encoding 95 amino acids of the Na,K-ATPase α1-subunit NH2-terminus was amplified by AmpliTaq Gold (Applied Biosystems). The PCR product was cloned in pENTR/D-TOPO vector using pENTR Directional TOPO Cloning Kit (Invitrogen) and subcloned into pDEST-15 using Gateway System (Invitrogen) to obtain pGST-NKAα1.N95.

The nucleotide sequences of all constructs were confirmed by automated sequencing (KISEQ, Core Facilities of Karolinska Institutet, Stockholm, Sweden) and subsequent bioinformatics analysis using Lasergene software (DNASTAR).
For FRET control experiments, the cytosolic NH$_2$-terminus of aquaporin-4 (AQP4) was tagged with GFP to obtain pGFP-AQP4 (9). InsP$_3$R type 1 ligand binding protein (226-604 amino acids) with point mutation (R441Q) encoding InsP$_3$-sponge was cloned into a pEF-BOS-MCS vector (pEF-GSTm49-IRES-GFP) (10). pEGFP-actin was from Clontech.

**Cell Culture and Transfections**

Three types of renal cells were used: primary cultures of rat RPT cells prepared as described (11), COS-7 cells, a cell line derived from fetal monkey kidney, and LLC-PK$_1$ cells, a cell line derived from pig kidney. GFP-NKA$\alpha_1$ was stably expressed in COS-7 cells (12). Either pGFP-NKA$\alpha_1$, pGFP-NKA$\alpha_1$.M32, pEF-GSTM49-IRES-GFP, or pEGFP-actin was transiently transfected into RPT cells on culture day two using CLONfectin (Clontech). pGFP-AQP4 was transiently transfected into COS-7 cells on culture day two using CLONfectin (Clontech).

**Reagents**

Reagents were used at the following concentrations: cyclopiazonic acid (CPA) 20 $\mu$M, cytochalasin D (CytD) 5 $\mu$M, ouabain 100 pM - 250 $\mu$M, 2-aminoethoxydiphenylborate (2-APB) 5 $\mu$M, bradykinin 0.5 $\mu$M, and U73122 5 $\mu$M. All reagents were from Sigma.

**Intracellular Calcium and Sodium Measurements**

Intracellular Ca$^{2+}$ and Na$^+$ measurements were performed using Fura-2/AM and SBFI/AM (Molecular Probes), respectively, as previously described (2,11). After baseline recording, cells were treated and ratio images were recorded every 30 sec for 45-90 min. In each dish, 20-30 individual cells from a single cluster of cells were
analyzed. Results presented are representative single cell traces obtained from a minimum of 12 experiments.

**Immunocytochemistry and Confocal Microscopy**

For co-immunolocalization and FRET studies, COS-7 cells stably expressing GFP-NKAα1 were fixed with acetone for 3 min at room temperature and then incubated with PBS containing 5% (v/v) normal goat serum and 3% (w/v) BSA for 1 hour. InsP3Rs were probed with monoclonal mouse anti-rat InsP3R type 2 (KM1083) or type 3 (KM1082) antibodies (1 µg/ml) overnight at 4°C. Cy3 conjugated goat anti-mouse IgG antibody served as secondary antibody (1:1000, Jackson Immuno Research Laboratories). Cells were scanned with a Leica TCS SP inverted confocal scanning laser microscope.

NF-κB activation was measured in RPT cells by immunocytochemical staining as previously described (2). RPT cells transiently transfected with pGFP-NKAα1.M32 were treated with 250 µM ouabain for 30 min and then fixed using 3% paraformaldehyde (10 min). Following blocking as described above, cells were incubated with NF-κB p65 antibody (1:200, Santa Cruz Biotechnology) for 1 hour and then with Alexa 546 fluorescent secondary antibody (1:500, Molecular Probes) for 30 min. Slides were scanned using a Leica TCS SP inverted confocal scanning laser microscope and images of cells expressing the construct were identified by GFP signal; NF-κB immunostaining of cells was captured for the same field of view. NF-κB activation in individual cells was semi-quantitatively estimated by measuring the ratio between the mean NF-κB immunosignal in a given comparable area in the nucleus and cytoplasm in cells expressing GFP-NKAα1.M32 or those adjacent cells not expressing the construct using ImageJ (Wayne Rasband, National Institutes of Health).
FRET

Fluorescent resonance energy transfer (FRET) measurements were performed on a Leica TCS SP inverted confocal scanning laser microscope using a 40x/1.4 NA objective. A detailed description of the FRET technique can be found elsewhere (14,15). The Förster constant, $R_0$, for the donor-acceptor pair, GFP and Cy3, used in this study was 6 nm (16). FRET occurs when the fluorophores are separated by distances $0.5R_0 < r < 2R_0$. Thus, it is possible to distinguish proteins that are spatially co-localized within a 12 nm radius. To determine FRET, we quantified the quenching of donor fluorescence by performing acceptor photobleaching (14). COS-7 cells stably expressing GFP-NKA$\alpha$1 and stained with Cy3-labeled secondary goat anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories) to detect mouse monoclonal antibody to InsP$_3$R2 (KM1083) and InsP$_3$R3 (KM1082) were excited with 488 nm and 543 nm and collected separately. The acceptor, Cy3, was then irreversibly photobleached in a selected adequate region by continuous excitation with 543 nm and 633 nm lasers for 30-90 sec. Thereafter, the residual Cy3 and GFP image was obtained and identical regions, at the plasma membrane on individual cells, were outlined in the photobleached area and processed using ImageJ (Wayne Rasband, National Institutes of Health). Ratios between GFP-intensities of the plasma membrane region, after and before photobleaching, were calculated to quantify FRET. The FRET values presented are corrected for erroneous intensity changes in a selected region outside the bleached area. In a typical experiment, ten to fifteen cells were measured for each sample.

Immunoprecipitation Studies

Cells were solubilised in lysis buffer [50 mM Tris/HCL (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1% Triton X100, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche)] and left for 30 min on ice. Cell lysates were sonicated (3 X
2 sec at setting 2 using a Branson Sonifier 250, Branson Ultrasonics) and centrifuged at 9,000g at 4°C to obtain a crude cell extract. 500 µg of supernatant protein per reaction were precleared with protein G-sepharose for 1 hour to reduce background that may be caused by non-specific adsorption of cellular debris. After a low speed centrifugation, the resultant supernatant was incubated for 1 hour at 4°C with either a mouse monoclonal anti-Na,K-ATPase α1-subunit antibody (1:250; Upstate Biotechnology), a mouse monoclonal anti-InsP3R2 antibody (1:50; Santa Cruz Biotechnology) or a mouse monoclonal antibody anti-InsP3R3 antibody (1:50; BD Biosciences). Immunocomplexes were incubated with protein G-Sepharose beads overnight at 4°C. Beads were pelleted, washed, incubated with 2x Laemmli buffer and supernatants were subjected to SDS-gel electrophoresis using 6% acrylamide gels. Membranes were incubated overnight with a mouse monoclonal anti-InsP3R3 antibody (1 µg/ml; KM1082) and then for 1 hour using a horseradish peroxidase-conjugated secondary antibody (1:5000) prior to detection using ECL plus (Amersham Pharmacia Biotech). The resultant protein bands were scanned digitally and densitometrically analyzed by BioRad QuantitativeOne software.

**GST-pull Down Assay**

GST-NKAα1.N95 was produced in the BL21 strain of *Escherichia coli* and purified with glutathione-Sepharose 4B beads (Amersham Biosciences). Non-recombinant GST was used as a control. Detergent-extracted RPT cell lysate (prepared as described above for the co-immunoprecipitation protocol) was added to the beads in a 5 to 1 ratio (v/v) and incubated overnight at 4°C with gentle rotation. Beads were washed and re-suspended in 2x Laemmli buffer prior to SDS-gel electrophoresis (6% gel) and immunoblotting for InsP3,R3.
**Data Presentation and Analysis**

Data are presented as means ± SEM (standard error of mean) of a minimum of ten experiments, unless indicated otherwise. Student's $t$-test was used and significance was accepted at $p < 0.05$.

**Results**

In accordance with previous observations (2), ouabain (250 µM) induced highly regular intracellular Ca$^{2+}$ oscillations with a periodicity in the minute range in RPT cells (Fig. 1a). Typical Ca$^{2+}$ oscillations were detected about 5 to 15 min after ouabain exposure in approximately one-third of the cells and were generally initiated in one cell at the periphery of a cell cluster. Quantitatively and qualitatively, COS-7 cells treated with ouabain showed a similar Ca$^{2+}$ oscillatory response (Fig. 1b). Spontaneous oscillations in cytosolic Ca$^{2+}$ were never observed in untreated cells. Na,K-ATPase activity is dose-dependently inhibited by ouabain and 250 µM ouabain causes approximately 50% inhibition of rat Na,K-ATPase activity (2). Ouabain, 250 µM, exceeds circulating levels in rat, estimated to be in the pM-nM range (17). When cells were exposed to physiological ouabain doses (100 pM), Ca$^{2+}$ oscillations were observed (Fig. 1c) in approximately 1% of cells. For subsequent experiments designed to explore the mechanism by which Na,K-ATPase triggers Ca$^{2+}$ oscillations, we used 250 µM ouabain.

To elucidate the source of the Ca$^{2+}$ oscillatory response, intracellular endoplasmic reticulum (ER) Ca$^{2+}$ stores were depleted by pre-incubation with a sarco-endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA) (Fig. 1d). Ouabain did not induce Ca$^{2+}$ oscillations in CPA pre-treated cells. Regulated Ca$^{2+}$ release from intracellular ER Ca$^{2+}$ stores occurs mainly via InsP$_3$Rs or via ryanodine
receptors. InsP₃Rs are abundantly expressed in RPT cells whereas ryanodine receptors do not have any functional importance for ouabain-triggered Ca²⁺ oscillations in these cells (2). The membrane-permeable substance, 2-aminoethoxydiphenyl borate (2-APB), was initially introduced as a specific inhibitor of InsP₃Rs (18). The IC₅₀ for inhibition of InsP₃R-evoked Ca²⁺ release was reported to be 1-20 µM. Since then 2-APB has, in addition to its inhibitory effect on InsP₃-induced Ca²⁺ release, been shown to block store operated calcium (SOC) mediated cytosolic Ca²⁺ influx (19). SOC is generally fully inhibited by 50-100 µM 2-APB. Exposure of cells to concentrations of 2-APB higher than 100 µM may also cause a pronounced increase of basal cytosolic Ca²⁺, consistent with inhibition of the SERCA pump (19). Since available data suggests that low concentrations (up to 20 µM) of 2-APB will preferentially inhibit InsP₃Rs, we tested the effect of 5 µM 2-APB. Using this concentration, we found that ouabain-induced Ca²⁺ oscillations were abolished in the majority of cells treated with 2-APB (5 µM) (Fig. 1e). Collectively, the inhibitory effects of CPA and 2-APB demonstrate that release of Ca²⁺ via InsP₃R is an essential contributor to the Ca²⁺ oscillations triggered by the ouabain/Na⁺,K⁺-ATPase complex.

Activation of InsP₃Rs is critically dependent on activation of phospholipase C (PLC), phosphatidylinositol lipid hydrolysis and liberation of InsP₃. Notably however, recent studies indicate that InsP₃R function is also modulated by interaction with accessory proteins (20-22). To examine the role of InsP₃ for the ouabain-induced Ca²⁺ oscillations, RPT cells were transfected with a construct encoding a hyper-affinity InsP₃ absorbent, an InsP₃-sponge. The InsP₃-sponge, having more than a thousand-fold higher affinity for InsP₃ than InsP₃R, traps InsP₃ and abrogates InsP₃-induced Ca²⁺ release (10). The construct also encoded GFP to facilitate identification of transfected cells. Ouabain triggered low frequency Ca²⁺ oscillations in one-third of the cells expressing the InsP₃-
sponge (Fig. 1f). The amplitude of the oscillatory response was attenuated in some, but not all, of the cells expressing the InsP₃-sponge. To confirm the efficiency of the InsP₃-sponge in quenching InsP₃-mediated Ca²⁺ signaling in RPT cells, we treated cells with bradykinin, a well-known activator of PLC and InsP₃ production (23). Bradykinin induced single Ca²⁺ transients in virtually all non-transfected cells but was without effect in all cells expressing the InsP₃-sponge (Fig. 1g). Cells expressing only GFP exhibited regular ouabain-induced Ca²⁺ oscillations (data not shown). It was further found that pre-incubation of RPT cells with a PLC inhibitor, U73122, abolished bradykinin-induced Ca²⁺ transients (data not shown) but did not influence ouabain-induced Ca²⁺ oscillations (Fig. 1h). These findings indicate that ouabain-induced Ca²⁺ oscillations do not require increased InsP₃ levels to activate InsP₃R in this model.

Immunocytochemical studies, performed on COS-7 cells, revealed partial co-localization of Na,K-ATPase with InsP₃R type 1, 2 and 3 (InsP₃R₁, InsP₃R₂ and InsP₃R₃) respectively. Only InsP₃R₂ (Fig. 2a) and InsP₃R₃ (Fig. 2b) were studied in subsequent experiments since these isoforms were more abundantly expressed than InsP₃R₁. To investigate the spatial relationship between Na,K-ATPase and InsP₃R on a nanometer scale, FRET measurements were performed. In this protocol, we used COS-7 cells stably expressing GFP-tagged Na,K-ATPase α₁-subunit. These cells express approximately the same level of Na,K-ATPase as wild type COS-7 cells (12). GFP, which was fused to the cytosolic NH₂-terminus of Na,K-ATPase served as FRET donor (GFP-NKAα₁). The primary antibodies against InsP₃R₂ or InsP₃R₃ were probed with a Cy3-conjugated IgG secondary antibody, which served as the FRET acceptor (InsP₃R-Cy3). The epitopes recognized by the InsP₃R₂ and InsP₃R₃ antibodies are located in the cytoplasmic COOH-terminus of the respective InsP₃Rs (13). The GFP-NKAα₁ fluorescence intensity was, following acceptor photobleaching, enhanced 12.5±0.9% for
InsP$_3$R2 and 15.5±2.0% for InsP$_3$R3 (Fig. 3a,b). These results imply that the donor and acceptor complexes, GFP-NK$\alpha$1 and anti-InsP$_3$R-anti-mouse IgG-Cy3, were separated less than 12 nm, i.e. the maximal distance for FRET detection between GFP and Cy3 (16). Ouabain treatment significantly increased FRET (from 15.5±2.0% to 25.0±1.6%) between Na,K-ATPase and InsP$_3$R (Fig. 3a,b).

To confirm that the observed FRET between Na,K-ATPase and InsP$_3$R3 was a unique property of this pair of proteins and not merely the result of non-specific experimental artifacts, we designed control experiments using another integral plasma membrane protein, namely aquaporin-4 (AQP4). For these negative control experiments, GFP-AQP4 was expressed in COS-7 cells. FRET analysis was performed using GFP-AQP4 (donor) and the same Cy3-labeled secondary antibody to detect the InsP$_3$R3 antibody (InsP$_3$R3-Cy3, acceptor). No change in donor emission ratio before and after acceptor photobleaching was found for this molecular pair (data not shown). This result indicates that FRET between Na,K-ATPase and InsP$_3$R3 is not likely a result of non-specific effects of the fixation protocol on plasma membrane and ER membrane integrity and strengthens the conclusion that the physical association between Na,K-ATPase and InsP$_3$R3 is specific.

Co-immunoprecipitation studies added further support to the concept that Na,K-ATPase and InsP$_3$R are linked together in a microdomain. As shown in Fig. 3c, InsP$_3$R3 co-immunoprecipitated with Na,K-ATPase in COS-7 cells. The amount of InsP$_3$R3 that co-immunoprecipitated with Na,K-ATPase represented only a fraction (<50%) of the total InsP$_3$R3 present in the initial cell lysate. Non-immune IgG did not co-immunoprecipitate a detectable amount of InsP$_3$R3 (data not shown). Incubation of COS-7 cells with ouabain significantly increased the amount of InsP$_3$R3 associated with immunoprecipitated Na,K-ATPase (Fig. 3c,d). The propensity of InsP$_3$R3 to co-
immunoprecipitate with Na,K-ATPase was also demonstrated in RPT and LLC-PK₁ cells (Fig. 3e). InsP₃R isoforms form heterotetrametric channels (24), and as expected, InsP₃R2 co-immunoprecipitated with InsP₃R3 in all cell types (Fig. 3e).

Both Na,K-ATPase and InsP₃R bind to cytoskeleton proteins that are anchored by the actin network (25,26). To examine whether the signaling function of the Na,K-ATPase/InsP₃R complex depends on an intact cytoskeleton, RPT cells were pre-treated with cytochalasin D (CytD) to de-polymerize the actin cytoskeleton (Fig. 4a). Ouabain-induced Ca²⁺ oscillations (Fig. 4b), and FRET between Na,K-ATPase and InsP₃R3 (Fig. 4c,d) were completely abolished in CytD pre-treated cells. Also, no InsP₃R3 co-immunoprecipitated with Na,K-ATPase in cells pre-treated with CytD (Fig. 4e). Bradykinin induced normal single Ca²⁺ transients in cells pre-treated with CytD (data not shown).

The NH₂-terminus of Na,K-ATPase α₁-subunit is a flexible part of the molecule (27,28). We therefore initiated a series of experiments to test the cellular consequences of Na,K-ATPase NH₂-terminal truncation. RPT cells were transfected with a Na,K-ATPase α₁-subunit mutant, where 32 amino acids from the NH₂-terminus were deleted (NKAα₁.M32) (Fig. 5a). To identify transfected cells, NKAα₁.M32 was tagged with GFP (GFP-NKAα₁.M32). GFP-NKAα₁.M32 was localized predominantly at the plasma membrane (Fig. 5b). This limited truncation was chosen because it does not significantly impact Na,K-ATPase function; truncation of 32 amino acids from the NH₂-terminus results in a functional enzyme that possesses similar Na⁺/K⁺ exchange properties when transfected into cells (29). To confirm the function of the truncated enzyme on single cells, the effect of ouabain on intracellular Na⁺ concentration was monitored. Ouabain caused a similar increase in Na⁺ in cells expressing GFP-NKAα₁.M32 and cells that only expressed endogenous Na,K-ATPase (Fig. 5c).
indicating not only that the mutant was a fully functioning enzyme, but that it also preserved its capacity to bind ouabain. Ouabain-induced Ca\textsuperscript{2+} oscillations were not observed in GFP-NKA\textalpha{}1.M32 expressing cells (Fig. 5d), where as cells expressing only endogenous Na,K-ATPase \textalpha{}1-subunit did oscillate. Cells transiently transfected with a GFP-tagged full length Na,K-ATPase \textalpha{}1-subunit exhibited ouabain-triggered Ca\textsuperscript{2+} oscillations to the same extent as non-transfected cells (data not shown). These results indicate that the NH\textsubscript{2}-terminus of Na,K-ATPase \textalpha{}1-subunit plays a central role for induction of ouabain-induced Ca\textsuperscript{2+} oscillations. We also performed a GST-pull down assay where GST was fused to the full length (95 aa) NH\textsubscript{2}-terminus of the Na,K-ATPase \textalpha{}1-subunit. GST-NKA\textalpha{}1.N95 pulled down InsP\textsubscript{3}R3 from a lysate of RPT cells, while GST alone did not pull down InsP\textsubscript{3}R3 (Fig. 5e).

It was previously demonstrated that NF-\kappa{}B, a well-known Ca\textsuperscript{2+} dependent transcription factor, is more readily activated by low frequency Ca\textsuperscript{2+} oscillations than by a sustained Ca\textsuperscript{2+} increase (30). To exploit this effect and to determine the downstream functional implications of disturbing the communication between the NH\textsubscript{2}-terminus of Na,K-ATPase \textalpha{}1-subunit and InsP\textsubscript{3}R, we compared NF-\kappa{}B responsiveness to ouabain in cells expressing GFP-NKA\textalpha{}1.M32 with neighboring cells that only expressed the endogenous Na,K-ATPase \textalpha{}1-subunit. Fig. 6a shows a GFP-NKA\textalpha{}1.M32 expressing cell and Fig. 6b shows NF-\kappa{}B staining of this cell and its neighboring, untransfected cells in the same field of view following ouabain treatment for 30 min. NF-\kappa{}B activation was semi-quantitatively estimated by measuring the ratio of NF-\kappa{}B nuclear signal to cytosolic signal for each cell in the same field of view. Results from this analysis indicated that ouabain caused nuclear translocation of NF-\kappa{}B in non-transfected cells, but was without effect in GFP-NKA\textalpha{}1.M32 expressing cells (Fig. 6c). These findings demonstrate that truncation of the NH\textsubscript{2}-terminus of Na,K-ATPase \textalpha{}1-
subunit results in a functional Na,K-ATPase that resides at the plasma membrane, yet is sufficient to disrupt ouabain-induced activation of NF-κB.

Discussion

It is now generally agreed that many - if not most - important processes in the cell are controlled by proteins aggregated together in complexes (21,22,31-33). The assembly of complexes that contain a receptor and components of signal machinery provides the cell with a highly selective means to engage a specific signaling pathway. The finding that ligand-bound Na,K-ATPase assembles with InsP$_3$R and that this assembly can give rise to intracellular Ca$^{2+}$ oscillations with a constant periodicity in the minute range represents a novel principle for such a protein complex.

Based on our combined results, we suggest that the ouabain-induced Ca$^{2+}$ oscillation and signal transducing function of Na,K-ATPase is made possible by the local organization of Na,K-ATPase and InsP$_3$R into a spatially organized functional microdomain that links the plasma membrane to intracellular ER Ca$^{2+}$ stores. A signaling microdomain can function without a diffusible messenger provided the transducer and the effector are in such proximity that they can communicate via protein-protein interaction, either directly, or via one or more interacting/scaffolding proteins. Molecular strategies using the InsP$_3$-sponge and pharmacological studies using the PLC inhibitor, U73122, indicate that the ouabain-induced Ca$^{2+}$ oscillatory response may be elicited via an InsP$_3$-independent mechanism of InsP$_3$R activation. Significant energy transfer between the donor and acceptor complexes, GFP-NKA$\alpha$1 and anti-InsP$_3$R-anti-mouse IgG-Cy3, indicate a distance of less than 12 nm (16). It is well established that the ER is juxtaposed to the plasma membrane (34). FRET was recorded in a region of the plasma membrane and the result is well compatible with previous electron
microscopy studies showing that the distance between the plasma membrane, where Na,K-ATPase is located, and the membrane of ER, where the InsP₃R is located, can be as short as 10 nm (35). It should be noted though, that FRET is recorded between the GFP labeled Na,K-ATPase and Cy3 labeled goat-anti-mouse IgG antibody that binds to the InsP₃R antibody. Taking the size of the antibodies and the GFP molecule into account, the maximal distance between Na,K-ATPase and InsP₃R could be somewhat larger than the working distance for FRET fluorophores, GFP and Cy3. Hence, the FRET results do not rule out the possibility that Na,K-ATPase and InsP₃R may interact via a scaffolding protein. The observed increase in FRET between Na,K-ATPase and InsP₃R following ouabain exposure and the loss of FRET upon CytD treatment indicate that ouabain-induced Ca²⁺ oscillations were dependent on a dynamic physical association between Na,K-ATPase and InsP₃R and therefore add further evidence to the concept that the Ca²⁺ signal arises from a signaling microdomain containing Na,K-ATPase and InsP₃R.

The question remains as to how the localization of Na,K-ATPase and InsP₃R is controlled. As evidenced by our FRET and co-immunoprecipitation studies using CytD, an intact cytoskeleton is required for the physical association between Na,K-ATPase and InsP₃R and ouabain-induced Ca²⁺ signaling. This suggests that actin, or cytoskeletal proteins associated with actin, will stabilize the Na,K-ATPase/InsP₃R complex by physical tether cross-linking. Ankyrins, a family of adaptor proteins believed to participate in the organization of proteins into specialized regions in the plasma membrane and ER Ca²⁺ stores, represent an interesting putative mechanistic partner for orchestrating Na,K-ATPase and InsP₃R proximity (25). The complete framework of adaptor and scaffolding proteins that may be involved in facilitating the structure and signal transducing function of this microdomain is still uncertain, but our results also do
not preclude the necessity of a direct physical interaction between Na,K-ATPase and InsP₃R in this event. The activation of InsP₃R is likely due to an allosteric effect of ouabain on Na,K-ATPase. Na,K-ATPase is a P-type ATPase that can exist in distinct E₁ and E₂ conformational states that are at least partially determined by intramolecular interactions between the NH₂-terminus and cytoplasmic loops of the enzyme. Ouabain binds to the E₂ conformation of the Na,K-ATPase and causes a robust shift in the E₁/E₂ poise towards E₂ forms (36). Information derived from the crystal structure of another P-type ATPase, the SERCA pump, suggests that the E₁-E₂ state transition is accompanied by significant movement of the three cytoplasmic domains, N (nucleotide binding), P (phosphorylation) and A (actuator) (37). A recent study (28) suggests that the NH₂-terminus may act as an auto-regulatory domain, modulating E₁/E₂ conformational transition. In E₁ conformation, the NH₂-terminus is in association with the first cytoplasmic loop of Na,K-ATPase. Transition from E₁ to E₂ conformation may release the NH₂-terminus from its interaction with the first cytoplasmic loop of Na,K-ATPase, thus making the NH₂-terminus available for interaction either with the InsP₃R directly or with a protein bridging between Na,K-ATPase and InsP₃R. Such an effect could explain why truncation of the first 32 amino acids of the NH₂-terminus will prevent ouabain-induced Ca²⁺ oscillations. An alternative explanation is that NH₂-terminal truncation has displaced the E₁-E₂ conformation of Na,K-ATPase in favor of E₁ (8,38).

The concept that Na,K-ATPase may, in addition to its function as an ion pump, also act as a signal transducer, is now rapidly evolving (2-5). Most previous studies on the signaling role of Na,K-ATPase have been carried out on cardiac myocytes (for review see (39)). In these cells, activation of Src kinase was found to be the primary event in the signaling cascade initiated by the ouabain/Na,K-ATPase complex, and downstream
effects included phosphorylation of epidermal growth factor receptor and activation of the mitogen activated protein kinase pathway. Since cardiac myocytes exhibit spontaneous Ca\(^{2+}\) sparks of high frequency, it has not yet been possible to establish whether Na,K-ATPase/InsP\(_3\)R triggered Ca\(^{2+}\) oscillations also occur in these cells. Another model where Na,K-ATPase mediated Ca\(^{2+}\) signaling is dependent on a close proximity between the plasma membrane and ER Ca\(^{2+}\) stores has previously been presented (40). In this model, Na,K-ATPase \(\alpha_2\)- and \(\alpha_3\)-subunits modulate Ca\(^{2+}\) release from ER via local changes in intracellular Na\(^+\) concentration. Our study was performed on cells expressing only the \(\alpha_1\)-subunit of Na,K-ATPase and the results from the protocols with the truncated NKA\(\alpha_1\).M32 imply that increased intracellular Na\(^+\) concentration was, in our model, not the main cause of ouabain-induced Ca\(^{2+}\) oscillations. Furthermore, we previously established that increasing intracellular Na\(^+\), by lowering extracellular K\(^+\), to the same extent as 250 \(\mu\)M ouabain, is not sufficient to elicit a Ca\(^{2+}\) oscillatory response (2).

Ca\(^{2+}\) oscillations trigger such universal processes as fertilization, cell division, cell differentiation and apoptosis (41). The specificity of this signal is encoded by the frequency and/or the amplitude of the Ca\(^{2+}\) oscillations (42). In the case of G-protein coupled receptors, Ca\(^{2+}\) oscillations generally have a periodicity of seconds (30,43). The highly regular ouabain/Na,K-ATPase-induced Ca\(^{2+}\) oscillations displayed a longer periodicity in the minute range, and should thereby provide a mechanism to ensure a different kind of physiological read-out.

Endogenous ouabain levels are high during pregnancy and in the perinatal period (44,45). Thus, it is of great physiological interest that the ouabain-induced slow Ca\(^{2+}\) oscillations were found to activate NF-\(\kappa\)B. This pluri-potent transcription factor activates genes modulating cell proliferation, apoptosis and development as well as
responses of the immune system (46). Cells expressing GFP-NKAα1.M32 did not respond to ouabain with Ca\textsuperscript{2+} oscillations. In these cells ouabain did not activate NF-κB. These findings support the physiological significance of a signaling microdomain containing Na,K-ATPase α1-subunit and InsP\textsubscript{3}R as a trigger mechanism for NF-κB activation.

In conclusion, we have demonstrated the existence of a cell signaling microdomain containing Na,K-ATPase and InsP\textsubscript{3}R that acts as a signaling pathway for Ca\textsuperscript{2+} oscillations and activation of NF-κB. Our findings represent, in many aspects, a novel mechanism for generation of Ca\textsuperscript{2+} signaling where an ion pump, Na,K-ATPase, also functions as a signal transducer. The physiological importance of slow Ca\textsuperscript{2+} oscillations is generally recognized, yet few biological generators of this signal have previously been identified.

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References

Fig. 1. **Intracellular Ca\(^{2+}\) response to ouabain in renal cells.** Ca\(^{2+}\) oscillations in (a) 250 µM ouabain treated RPT cells, (b) 250 nM ouabain treated COS-7 cells, (c) 100 pM ouabain treated RPT cells. (d) CPA depleted intracellular ER Ca\(^{2+}\) stores and abolished ouabain-induced Ca\(^{2+}\) oscillations in RPT cells. Each trace represents a single cell recording. (e) Ouabain-induced Ca\(^{2+}\) oscillations in RPT cells were abolished by 2-APB (5 µM). (f) Two representative single cell recordings of cytosolic Ca\(^{2+}\) in RPT cells transfected with the InsP\(_3\)-sponge (n = 15). The amplitude was lower in some InsP\(_3\)-sponge expressing cells. (g) Two representative single cell recordings of cytosolic Ca\(^{2+}\) in RPT cells. Bradykinin did not induce Ca\(^{2+}\) transients in InsP\(_3\)-sponge expressing RPT cells (trace A) while non-transfected cells exhibited Ca\(^{2+}\) transients (trace B). (h) U73122 (5 µM) did not abolish ouabain-induced Ca\(^{2+}\) oscillations in RPT cells. Arbitrary units (a.u.) represent ratio values corresponding to intracellular Ca\(^{2+}\) concentration changes.

Fig. 2. **Immunocytochemical studies of Na\(_k\),K-ATPase and InsP\(_3\)R localization in COS-7 cells.** Na\(_k\),K-ATPase (GFP-NKA\(_{\alpha_1}\)) and (a) InsP\(_3\)R2 (InsP\(_3\)R2-Cy3) or (b) InsP\(_3\)R3 (InsP\(_3\)R3-Cy3) co-localize near the plasma membrane.

Fig. 3. **Studies of Na\(_k\),K-ATPase and InsP\(_3\)R signaling microdomain.** (a,b) FRET measurements between Na\(_k\),K-ATPase and InsP\(_3\)R3. (a) GFP-NKA\(_{\alpha_1}\) images of COS-7 cells with and without ouabain treatment before and after acceptor photobleaching (bleached area indicated by square). (b) Quantitative changes in emission intensities after bleaching compared to before bleaching, mean ± SEM, * p < 0.05. FRET was enhanced by ouabain. (c-e) Co-immunoprecipitation (IP) studies followed by Western blotting (WB) for InsP\(_3\)R3. (c) Representative Western blot and (d) densitometric
analysis of InsP$_3$R3 content in Na,K-ATPase immunoprecipitates before and after 250 µM ouabain treatment for 30 min in COS-7 cells. Ouabain significantly increased the amount of InsP$_3$R3 associated with Na,K-ATPase, mean ± SEM (n = 3), * $p < 0.05$. Molecular weight markers are indicated to the left of the blot. (e) InsP$_3$R3 co-immunoprecipitated with Na,K-ATPase and InsP$_3$R2 in COS-7, RPT and LLC-PK$_1$ cells.

Fig. 4. **Effect of cytoskeleton perturbation on physical association between Na,K-ATPase and InsP$_3$R.** (a) Actin cytoskeleton was disrupted after CytD (5 µM) treatment in GFP-actin expressing RPT cells. (b) CytD abolished ouabain-induced Ca$^{2+}$ oscillations in RPT cells. Arbitrary units (a.u.) represent ratio values corresponding to intracellular Ca$^{2+}$ concentration changes. (c,d) FRET measurements between Na,K-ATPase and InsP$_3$R3. (c) GFP-NKA$\alpha$1 images of COS-7 cells with and without CytD treatment before and after acceptor photobleaching (bleached area indicated by square). (d) Quantitative changes in emission intensities after bleaching compared to before bleaching, mean ± SEM, * $p < 0.05$. FRET was eliminated by CytD. (e) Co-immunoprecipitation (IP) studies followed by Western blotting (WB) for InsP$_3$R3 in CytD treated COS-7 cells. InsP$_3$R3 did not co-immunoprecipitate with Na,K-ATPase.

Fig. 5. **Role of NH$_2$-terminus of Na,K-ATPase $\alpha$1-subunit in ouabain-induced Ca$^{2+}$ signaling.** (a) NH$_2$-terminus of the rat Na,K-ATPase $\alpha$1-subunit and deletion mutant thereof. Numbering is based on the mature rat $\alpha$1-subunit amino acid sequence (47). (b) Confocal microscope image of a GFP-NKA$\alpha$1.M32 expressing RPT cell, recorded with a small pinhole to optimize membrane signal. (c) Intracellular Na$^+$ measurements following ouabain treatment (at time zero) in RPT cells expressing GFP-NKA$\alpha$1.M32 (trace A) and endogenous Na,K-ATPase $\alpha$1-subunit (trace B). (d) Single cell recordings of intracellular Ca$^{2+}$ response to ouabain in GFP-NKA$\alpha$1.M32
expressing RPT cells. No Ca\textsuperscript{2+} oscillations were observed. Arbitrary units (a.u.) represent ratio values corresponding to intracellular Ca\textsuperscript{2+} and Na\textsuperscript{+} concentration changes. (e) GST-pull down assay with RPT cell lysate followed by Western blotting for InsP\textsubscript{3}R3.

**Fig. 6. NF-κB translocation in GFP-NKA\textalpha{}1.M32 expressing cells following ouabain treatment.** (a) GFP-NKA\textalpha{}1.M32 expressing RPT cell identified by GFP fluorescence. Images were recorded with an open pinhole to measure semi-quantitatively the ratio between NF-κB abundance in the nucleus and cytosol. (b) NF-κB immunosignal of GFP-NKA\textalpha{}1.M32 expressing and non-expressing RPT cells, from same field of view as (a). (c) NF-κB activation in GFP-NKA\textalpha{}1.M32 expressing (n = 47) and non-expressing (n = 218) cells following ouabain treatment, mean ± SEM, *p < 0.05. Ouabain induced NF-κB nuclear translocation was abrogated in GFP-NKA\textalpha{}1.M32 expressing cells.
Figure 1, Miyakawa-Naito et al.
Figure 2, Miyakawa-Naito et.al.

a

GFP-NKA$_{\alpha 1}$  InsP$_3$R2-Cy3  Merged

b

GFP-NKA$_{\alpha 1}$  InsP$_3$R3-Cy3  Merged
Figure 3, Miyakawa-Naito et al.
Figure 4, Miyakawa-Naito et al.

(a) Before and After images.

(b) Graph showing changes in [Ca^2+](a.u.) over time (min) with Ouabain and CytD.

(c) FRET images before and after CytD treatment.

(d) Bar graph showing percent increase (%) with CytD.

(e) Western Blot showing InsP_3R1, InsP_3R2, and InsP_3R3.
Figure 5, Miyakawa-Naito et al.

a

NKA\(\alpha_1\) (MGKGV) GRDKYEPAAVSEHGDKSKKAKKRDDELKEVSMDDHKLSDLDELHRYGTDLSRGTLTPARAAEILARDPNALTPFPPTPEMVKFCRQ...

NKA\(\alpha_1\).M32

b

GFP-NKA\(\alpha_1\).M32

c

\([\text{Na}^+]_i\) (a.u.)

\(-10\) 0 10 20 30 40

Time (min)

d

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

0 10 20 30

Time (min)

e

1: GST-NKA\(\alpha_1\).N95

2: GST

1

2
Figure 6, Miyakawa-Naito et al.
Cell signaling microdomain with Na,K-ATPase and inositol 1,4,5-trisphosphate receptor generates calcium oscillations
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