Regulation of Inositol 1,4,5-Trisphosphate Receptor-mediated Calcium Release by the Na/K-ATPase in Cultured Renal Epithelial Cells*

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It is known that the Na/K-ATPase α1 subunit interacts directly with inositol 1,4,5-trisphosphate (IP₃) receptors. In this study we tested whether this interaction is required for extracellular stimuli to efficiently regulate endoplasmic reticulum (ER) Ca²⁺ release. Using cultured pig kidney LLC-PK1 cells as a model, we demonstrated that graded knockdown of the cellular Na/K-ATPase α1 subunit resulted in a parallel attenuation of ATP-induced ER Ca²⁺ release. When the knockdown cells were rescued by knocking in a rat α1, the expression of rat α1 restored not only the cellular Na/K-ATPase but also ATP-induced ER Ca²⁺ release. Mechanistically, this defect in ATP-induced ER Ca²⁺ release was neither due to the changes in the amount or the function of cellular IP₃ and P2Y receptors nor the ER Ca²⁺ content. However, the α1 knockdown did redistribute cellular IP₃ receptors. The pool of IP₃ receptors that resided close to the plasma membrane was abolished. Because changes in the plasma membrane proximity could reduce the efficiency of signal transmission from P2Y receptors to the ER, we further determined the dose-dependent effects of ATP on protein kinase Cε activation and ER Ca²⁺ release. The data showed that the α1 knockdown desensitized the ATP-induced ER Ca²⁺ release but not PKCe activation. Moreover, expression of the N terminus of Na/K-ATPase α1 subunit not only disrupted the formation of the Na/K-ATPase/IP₃ receptor complex but also abolished the ATP-induced Ca²⁺ release. Finally, we observed that the α1 knockdown was also effective in attenuating ER Ca²⁺ release provoked by angiotensin II and epidermal growth factor.

The Na/K-ATPase is a highly expressed integral membrane protein that hydrolyzes ATP to pump Na⁺ and K⁺ across the membrane (1). It also functions as an important signal transducer (2). Recent studies have demonstrated that cells appear to contain two functionally separable pools of Na/K-ATPase and that a majority of the cellular Na/K-ATPase is engaged in cellular activities other than pumping ions (3). Moreover, the non-pumping Na/K-ATPase apparently resides in caveolae and interacts directly with protein kinases, ion channels, and transporters (4). The interaction between the Na/K-ATPase and Src, for example, forms a functional receptor complex for cardiotonic steroids such as ouabain to activate protein tyrosine phosphorylation (5, 6). Interestingly, recent studies from several laboratories have demonstrated a direct interaction between the α subunit of Na/K-ATPase and inositol 1,4,5-trisphosphate receptors (IP₃Rs) (7–10). In addition, we have found that ouabain was capable of stimulating the formation of a functional Ca²⁺-signaling complex consisting of the Na/K-ATPase/Src/PLC-γ/PIP₂ in LLC-PK1 cells (9). Furthermore, we have shown that the formation of this signaling complex plays an important role in ouabain-induced Ca²⁺ signal transduction.

The cytosolic free calcium is one of the most important cellular second messengers. Calcium enters the cytosol via the opening of Ca²⁺ channels that either resides in the plasma membrane or in the membranes of intracellular organelles such as the ER. On the ER, IP₃Rs are widely expressed and play an important role in converting the activation of many plasma membrane receptors into intracellular Ca²⁺ signaling. To date, three isoforms of IP₃Rs have been identified, sharing similar but not identical functional properties (11). The signaling events leading to the ER Ca²⁺ release from these IP₃Rs are initiated by the activation of phospholipase C-coupled plasma membrane receptors such as the Na/K-ATPase and P2Y receptors and subsequent generation of IP₃, a ligand for IP₃Rs. It has been proposed that the formation of the junctional microdomains that force the proximity of IP₃Rs to the plasma membrane receptors provides a mechanism for defining spatially and temporally specific Ca²⁺ signaling (12–15). For instance, the forced coupling between B2 bradykinin receptors and IP₃Rs ensures a robust Ca²⁺ signaling when the receptor is activated by the ligand in neuronal cells (16). These findings raise the question as to whether there is a plasma membrane protein that can function as an anchor to interact and pull the ER IP₃Rs to the proximity of the plasma membrane receptors.

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‡ The abbreviations used are: IP₃, inositol 1,4,5-trisphosphate receptor; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor type 2; IP₃R, IP₃ receptor type 3; ER, endoplasmic reticulum; [Ca²⁺]ᵢ, intracellular calcium; PIP₂, phosphatidylinositol 4,5-biphosphate; DAG, 1,2-diacylglycerol; PLC, phospholipase C; eYFP, enhanced yellow fluorescence protein; PH domain, pleckstrin homology domain; EGF, epidermal growth factor; GFP, green fluorescent protein; PKC, protein kinase C; TG, thapsigargin; Ab, antibody.
It is well documented that many G protein-coupled receptors and receptor tyrosine kinases are concentrated in caveolae (17, 18). Because the Na/K-ATPase represents a highly abundant caveolar membrane protein, it is conceivable that the basal interaction between the caveolar Na/K-ATPase and ER IP3Rs could force the proximity of ER IP3Rs to other plasma membrane receptors. Thus, the interaction between the Na/K-ATPase and IP3Rs may not only be important for ouabain-induced ER Ca2+ release but also play a role in other stimuli-induced Ca2+ signaling. To test this hypothesis, we examined the effect of graded knockdown of Na/K-ATPase on ATP-induced ER Ca2+ release. The data presented here clearly demonstrated that the cellular Na/K-ATPase regulates ER Ca2+ release by interacting and targeting the IP3Rs in LLC-PK1 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fura-2 AM, Image-iT FX signal enhancer, antibody kit, Alexa Fluor 488-conjugated anti-mouse and anti-goat IgG, and Alexa Fluor 555-conjugated anti-mouse IgG antibodies were obtained from Molecular Probes (Eugene, OR). U73122 and U73343 were obtained from Cayman (Ann Arbor, MI). Sulfo-NHS-biotin, immobilized streptavidin-agarose beads were obtained from Pierce. Anti-P2Y2 receptor polyclonal antibody was obtained from Zymed Laboratories Inc.; anti-YFP polyclonal antibody was obtained from Abcam (Cambridge, MA); and anti-calnexin polyclonal antibody was obtained from StressGen Bioreagents Corp. (Victoria, British Columbia, Canada). The sources of other primary antibodies were described previously (9). All the horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Preparation and Culture**—LLC-PK1 cells and the Na/K-ATPase α1 knockout (e.g., A4-11, PY-17, and TCN23-19) and knock-in (AAC-19) cells were maintained in the growth medium as described previously (19). When cell cultures reached about 80% confluence, cells were serum-starved for 24 h and used for Western blot analysis.

**Plasmids and Transfection**—N-terminal (Ala1–Ser160) of the rat α1 was cloned from rat Na/K-ATPase α1 cDNA provided by Dr. Pressley (Texas Tech University) and inserted in-frame into pEYFP-N1 vector (Clontech). The construct was verified by DNA sequencing. For calcium imaging, LLC-PK1 cells were transfected with various plasmids using calcium phosphate precipitation (20). Experiments were performed 24 h after transfection unless indicated otherwise.

**Calcium Imaging in Live Cells**—Intracellular calcium concentration was measured as described previously (9). In brief, cells were incubated with 2 μM Fura-2 AM at 25 °C for 40 min in a physiological salt solution (ECB) containing 10 mM NaCl, 5 mM KCl, 20 mM HEPES, 25 mM NaHCO3, 1 mM CaCl2, 1.2 mM MgCl2, 1 mM Na2PO4, and 10 mM d-glucose. Coverslips were placed in a recording/perfusion chamber (model QE-1, Warner Instruments) mounted on the stage of an inverted microscope (model IX71, Olympus) equipped with a 40× oil-immersion Fluor objective. Excitation light alternated between 340 and 380 nm, and emitted light was recorded at >510 nm. Ratio images were acquired and analyzed using SlideBook software (SlideBook 4.1.0, Intelligent Imaging Innovations, Inc.). Cells were perfused with ECB at 37 °C at the speed of 0.5 ml/min, and then the solution was quickly changed to a nominally calcium-free ECB supplemented with 250 μM EGTA. Cells were excited for 2 ms every 5 s and monitored for less than 30 min.

**Assay of IP3 Production and PKCε Translocation**—GFP-fused PLC-δ1 PH domain (PHD-GFP) was used to monitor PIP3 hydrolysis and IP3 production in live cells as described previously (9). Cells were transfected with PHD-GFP in OptiMEM medium using Lipofectamine 2000. After 24 h, the cells were exposed to stimulus and monitored for changes in GFP signal in the plasma membrane and the corresponding cytosol.

To analyze PKC activation, cells were treated and then washed with ice-cold phosphate-buffered saline. Afterward, cells were collected in a buffer containing 10 mM EGTA, 1 mM EDTA, 0.5 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 25 μg/ml aprotinin, and 20 μg/ml Tris-HCl (pH 7.5) and homogenized in a Potter-Elvehjem homogenizer as described previously (21). The suspension was centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant was removed (the cytosolic fraction). The pellet was suspended in the above described buffer to which Triton X-100 (1%) was added and sonicated for 10 s. After 30 min of incubation on ice, the mixture was centrifuged at 25,000 × g for 10 min, and the supernatant was collected (the particulate fraction). To assay the translocation of PKCε, the cytosolic and the particulate fractions were subjected to immunoblot analysis as described before.

**Immunocytochemistry**—Cells on coverslips were fixed in 4% paraformaldehyde for 30 min at 25 °C. Then the cells were permeabilized in fresh phosphate-buffered saline containing 0.5% saponin for 10 min and blocked with Image-iT FX signal enhancer for 30 min at room temperature. The cells were then incubated with primary antibodies (mouse anti-IP3R Ab, goat anti-IP3R2 Ab, and anti-Na/K-ATPase α1 monoclonal Ab) at 4 °C overnight. They were washed and then incubated with suitable Alexa Fluor 488- and Alexa Fluor 555-conjugated secondary antibodies at room temperature for 1 h. Coverslips were mounted using an antifade kit. Image analyses were performed as described previously (6, 19).

**Biotinylation of Cell Surface Proteins**—The cells were washed twice with ice-cold phosphate-buffered saline and then biotinylated as described previously (3, 22). The biotinylated cells were then lysed with 1 ml of radioimmunoprecipitation buffer for 30 min at 4 °C. Cell lysates were cleared by centrifugation at 14,000 × g for 15 min, and the supernatants (500 μg) were mixed with 200 μl of streptavidin-conjugated agarose beads overnight at 4 °C to recover the biotinylated proteins. The recovered proteins and 50 μg cell lysates were subjected to Western blot analysis.

**Immunoprecipitation of IP3R3 after Transient Transfection**—LLC-PK1 cells were transiently transfected with eYFP or αNT-eYFP by calcium phosphate precipitation as described previously (20). After 24 h, cells were lysed in radioimmunoprecipitation buffer, and lysates were prepared as above. Immunoprecipitation of IP3R3 was conducted as described previously (9).

**Statistical Analysis**—All traces and immunoblots presented are representative of at least three separate experiments. All
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With Ca

W/O Ca

20 μM ATP

100 sec

CON 2 μM 10 μM

quantitative data are given as mean ± S.E. Comparisons between two groups were analyzed via the Student’s t-test, and significance was accepted at $p < 0.05$.

RESULTS

Knockdown of Na/K-ATPase Reduces ATP-induced Ca$^{2+}$ Release in LLC-PK1 Cells—Extracellular ATP is known to modulate Ca$^{2+}$ mobilization either through the activation of P2Y receptor coupled to a G protein, or through the direct activation of the inotropic P2X receptor. LLC-PK1 cells are known to express both P2X and P2Y receptors (23, 24). Thus, we first determined the properties of ATP-induced Ca$^{2+}$ mobilization in LLC-PK1 cells. As depicted in Fig. 1, A and B, when cells were superfused with a nominally Ca$^{2+}$-free solution, application of 20 μM ATP induced a robust Ca$^{2+}$ transient, which reached the peak value within 1 min and then declined toward the basal level. In the presence of extracellular Ca$^{2+}$, ATP stimulated a similar but prolonged Ca$^{2+}$ transient that apparently involved both Ca$^{2+}$ release and Ca$^{2+}$ influx. To further test whether the ATP-induced Ca$^{2+}$ transient in the absence of extracellular Ca$^{2+}$ was because of the activation of PLC, the cells were first treated with PLC inhibitor U73122 and then exposed to ATP. As depicted in Fig. 1C, addition of U73122 completely abolished the effect of ATP on intracellular Ca$^{2+}$. On the other hand, the negative control U73343 did not affect ATP-induced Ca$^{2+}$ release (data not shown). Thus, we performed the following studies in cells incubated in a nominally Ca$^{2+}$-free solution to assess the role of Na/K-ATPase in IP$_3$R-mediated ER Ca$^{2+}$ release.

Recently, we have generated several Na/K-ATPase α1 knockdown cell lines from LLC-PK1 cells that were transfected with an α1-specific small interfering RNA (19). Although A4-11 cells express about 44% of α1 subunit in comparison with the control LLC-PK1 cells; PY-17 cells contain less than 10% of α1 subunit (Fig. 2A). When these cells were exposed to 20 μM ATP and monitored for changes in intracellular Ca$^{2+}$, we observed a significant decrease in ATP-induced Ca$^{2+}$ release in both A4-11 and PY-17 cells (Fig. 2B). When the peak value was quantified (Fig. 2C), the effect of ATP on intracellular Ca$^{2+}$ was proportional to the amount of cellular Na/K-ATPase. Moreover, when PY-17 cells were rescued by a rat α1, expression of rat α1 not only restored cellular Na/K-ATPase (19) but also ATP-induced ER Ca$^{2+}$ release (Fig. 2, B and C). Taken together, these findings indicate that knockdown of cellular Na/K-ATPase attenuated the ATP-induced ER Ca$^{2+}$ release. Because this reduction was observed in several knockdown cell lines, was proportional to cellular Na/K-ATPase amounts, and could be rescued by expression of exogenous Na/K-ATPase, it was unlikely a cloning artifact.

Inhibition of Na/K-ATPase by Ouabain Does Not Attenuate ATP-induced Ca$^{2+}$ Release—We showed previously that Na/K-ATPase knockdown resulted in a significant inhibition of cellular sodium pump activity and a subsequent increase in intracellular Na$^+$ concentration (3). To rule out that the attenuation of ATP-induced Ca$^{2+}$ release is because of a simple inhibition of cellular Na/K-ATPase activity and subsequent increase in intracellular Na$^+$ concentration, we first compared the basal Ca$^{2+}$ in different cells. As shown in Fig. 2B, there was no detectable difference in basal Ca$^{2+}$ among these cell lines. Furthermore, when LLC-PK1 cells were treated with different concentrations of ouabain and then measured for ATP-induced Ca$^{2+}$ release, we found that neither 50% inhibition (1 μM) nor 100% inhibition (10 μM) of Na/K-ATPase by ouabain (25) changed ATP-induced Ca$^{2+}$ release in LLC-PK1 cells (Fig. 2D).

Na/K-ATPase Knockdown Does Not Affect Purinergic Receptor Function and ER Calcium Contents—To date, pharmacological characterization and molecular cloning have identified eight P2Y receptor subtypes, of which P2Y$_2$ receptor is highly expressed in renal epithelial cells (26). We showed that LLC-PK1 cells expressed both IP$_R$2 and IP$_R$3 (9). To test whether changes in the cellular amount of Na/K-ATPase affect the expression of P2Y$_2$ receptor and IP$_R$3, we performed Western blot analysis of the total cell lysates from control LLC-PK1 cells,
Interactions of Na/K-ATPase with IP3R

Interaction of Na/K-ATPase with IP3R

FIGURE 2. Effects of ATP on [Ca2+]i in Na/K-ATPase knockdown cells.

A, representative Western blots show the expression of the α1 subunit of Na/K-ATPase in different cell lines. Total cell lysates were 40 μg per lane. IB, immunoblot. B, cytoplasmic calcium was measured in parallel in different cell lines as in Fig. 1. Calcium traces show the transient elevation of [Ca2+]i, induced by 20 μM ATP. C, bar graph summarizes the peak cytosolic calcium elevation induced by ATP in all experiments (mean ± S.E.). *, p < 0.05; **, p < 0.01. D, partial (1 μM) or total (10 μM) inhibition of Na/K-ATPase by ouabain did not change ATP-induced Ca2+ release in LLC-PK1 cells. The cells were pre-exposed to ouabain for 5 min and then superfused with 20 μM ATP in the presence of ouabain. Data were from 12 coverslips (each condition has four repeats). CON, control.

The knockdown and knock-in cells. As shown in Fig. 3A, knockdown of Na/K-ATPase did not change the expression of these receptors.

Binding of ATP to P2Y receptor activates PLC-β, which subsequently catalyzes the production of second messengers diacylglycerol (DAG) and IP3. To determine whether Na/K-ATPase knockdown affected the P2Y receptor function, we measured the effect of ATP on DAG production using PKCε activation as an indicator. As shown in Fig. 3B, cells were stimulated with 20 μM ATP for 5 min. Analysis of PKCε translocation demonstrated a similar activation of PKCε by ATP in control LLC-PK1, Na/K-ATPase knockdown and knock-in cells (Fig. 3C).

To seek further support, we also measured ATP-induced IP3 production in live cells using a GFP-fused PLC-δ1 PH domain protein as a probe (27). The imaging analysis was conducted in the control LLC-PK1 cells and another α1 knockdown TCN23-19 cell line. Like PY-17 cells, TCN23-19 cells expressed about 10% of Na/K-ATPase in comparison with the control LLC-PK1 cells (19). Moreover, the ATP-induced ER Ca2+ release was similarly attenuated as in PY-17 cells (Fig. 3D).

Importantly, expression of rat α1 in TCN23-19 cells also restored the effect of ATP on ER Ca2+ release (data not shown). However, unlike PY-17 cells, these cells did not express YFP, making it easier to run GFP imaging analysis and immunostaining. As depicted in Fig. 3E, cells were transiently transfected with the vector expressing GFP-fused PLC-δ1 PH domain. In unstimulated cells, the PH domain bound to PIP2 and mainly associated with the plasma membrane. When PIP2 was hydrolyzed by PLC to produce IP3 in response to the addition of 20 μM ATP, the PH domain translocated into the cytoplasm together with IP3. This resulted in an increase in intensity.

FIGURE 3. P2Y receptor function and ER calcium levels were not affected by knockdown of Na/K-ATPase.

A, Western blot analysis of different receptors in the total cell lysates (30 μg) from different cell lines. IB, immunoblot. B, effects of ATP on the PKCε translocation. Cells were exposed to 20 μM ATP for 5 min and analyzed for PKCε translocation as described under “Experimental Procedures.” Representative blots are shown. C, cytosolic fraction; P, particulate fraction. C, PKCε translocation data are summarized from five independent experiments and normalized to the control of LLC-PK1 cells. **, p < 0.01. D, ATP-induced calcium release in TCN23-19 cells in comparison with LLC-PK1 cells. Representative calcium traces show the transient calcium elevation induced by 20 μM ATP. E, effect of ATP on PIP2 hydrolysis and IP3 production in LLC-PK1 cells and TCN23-19 cells. Confocal images show the expression of PLC-δ1 PHD-GFP in the plasma membrane. Traces show that the plasma membrane GFP signal (marked by a blue box) decreases with concomitant increases in cytosolic GFP signal (marked by a red box) in response to 20 μM ATP stimulation. F, cytoplasmic calcium elevation induced by 3 μM TG was measured in parallel in different cell lines. Bar graph summarizes the quantitative results measured in five independent experiments.
Interaction of Na/K-ATPase with IP$_3$R

Knockdown did not significantly change the ATP-induced IP$_3$ release. E, biotinylation of the plasma membrane IP$_3$R. Cells were biotinylated with sulfo-NHS-biotin as described under "Experimental Procedures." Streptavidin-precipitated samples and 10% of cell lysate inputs were subjected to SDS-PAGE, and then analyzed by Western blot with the monoclonal anti-IP$_3$R3 Ab and polyclonal anti-calnexin Ab. Representative immunoblots are shown. EX, total cell lysates; IB, Streptavidin-bound fraction. IB, immunoblot. F, combined data from five experiments are presented. The plasma membrane IP$_3$R3 expressed in LLC-PK1 cells is 1.82 ± 0.18% versus 1.88 ± 0.29% in PY-17 cells and 1.95 ± 0.16% in TCN23-19 cells.

Because anti-IP$_3$R3 antibody produced a much clearer cell periphery signal, we repeated the same staining of IP$_3$R in the TCN23-19 cells. Although the overall density of IP$_3$R in TCN23-19 cells was similar to that in the control LLC-PK1 cells, knockdown of the Na/K-ATPase caused a significant change in subcellular distribution of IP$_3$R3. Specifically, the cell periphery distribution of the IP$_3$R3 was totally abolished (Fig. 4D).

Because IP$_3$R could reside in the plasma membrane (22), the cell periphery distributed IP$_3$R may come from two pools as follows: one from the IP$_3$R in the plasma membrane and the other from the IP$_3$R in the ER membrane that is in close proximity to the plasma membrane. To distinguish these two pools, we performed the cell surface biotinylation study. Quantitative analysis indicated that about 1.8% of total cellular IP$_3$R3 was biotinylated in LLC-PK1 cells. Because the same procedure failed to detect any calnexin, a known 90-kDa integral ER membrane protein, in the biotinylated samples (Fig. 4E), the detected IP$_3$R3 most likely resided in the plasma membrane, which is consistent with the prior observations (22). When the same experiments were performed in both TCN23-19 and PY-17 cells, we detected the same amount of biotin-labeled IP$_3$R3 as in the control LLC-PK1 cells (Fig. 4, E and F). Thus, the disappearance of cell periphery IP$_3$R3 in TCN23-19 cells was likely because of the redistribution of IP$_3$R3s that resided in proximity to the plasma membrane.

**Na/K-ATPase Facilitates the Signal Transmission from the Plasma Membrane Receptors to ER IP$_3$R Receptors**—The above data suggest that the Na/K-ATPase may function as a plasma membrane scaffold, forcing the proximity and facilitating the coupling between the ER IP$_3$R and the plasma membrane P2Y receptor. Should this be the case, we would expect that exposure of PY-17 cells to a higher concentration of ATP might compensate for the deficiency in coupling of ER IP$_3$R to the P2Y receptor if higher concentration of ATP can activate additional receptors. To test this, we first compared the effects of 20, 50, and 100 μM ATP on PKCe activation and found that the activation of PKCe reached the maximal level by 50 μM ATP in both LLC-PK1 and PY-17 cells (Fig. 5, A and B). The effects of ATP on ER Ca$^{2+}$ release were also dose-dependent (Fig. 5C). Significant effect was observed when LLC-PK1 cells were exposed to 1 μM ATP, and the maximal effect was reached at 10 μM ATP. Thus, in LLC-PK1 cells, partial activation of the plasma membrane P2Y receptors by 10 μM ATP was sufficient to induce the maximal ER Ca$^{2+}$ release. However, when ATP concentration curve on ER Ca$^{2+}$ release was determined in PY-17 cells, we found that 50 μM ATP was required to produce the maximal Ca$^{2+}$ release (Fig. 5C). Moreover, the effect of 50 μM ATP on Ca$^{2+}$ release in PY-17 cells was only comparable with that produced by 1 μM ATP in LLC-PK1 cells. Finally, 1 μM ATP failed to elicit ER Ca$^{2+}$ release in PY-17 cells. To be sure that this decrease in ATP sensitivity is not a cloning artifact of PY-17 cells, we repeated the ATP concentration curve on ER Ca$^{2+}$
release in TCN23-19 cells, observing an essentially identical dose-response curve as in PY-17 cells (data not shown). Taken together, our data clearly indicate that cellular Na/K-ATPase plays an important role for efficient signal transmission from the activated plasma membrane P2Y receptors to the ER IP3R.

**N Terminus of the Na/K-ATPase Functions as a Dominant Negative Regulator of ATP-induced ER Ca2+ Release**—Results from recent studies indicate that the N terminus of the Na/K-ATPase α1 subunit binds to IP3R directly (9, 10). To detect whether the effect of Na/K-ATPase on ER Ca2+ release is because of the direct interaction between Na/K-ATPase and IP3R, we constructed a YFP fusion protein containing the N terminus (amino acids 1–160) (αNT-eYFP). As shown in Fig. 6A, the expression of αNT-eYFP or eYFP in LLC-PK1 cells was dependent on the amount of vectors used in the transfection. When intracellular Ca2+ was measured in the transfected cells (indicated by the eYFP signal), we found that expression of αNT-eYFP, but not eYFP, dose-dependently inhibited ATP-induced ER Ca2+ release. Complete inhibition was achieved when LLC-PK1 cells were transfected with 2.0 μg of αNT-eYFP (Fig. 6, B and C). As an additional control, the effects of ATP on ER Ca2+ release were also measured in cells that were cultured on the same coverslip but were not transfected (no detectable YFP signal). As expected, these untransfected cells showed intact ability to release ER Ca2+ in response to ATP stimulation (data not shown).

To further test if αNT-eYFP can disrupt the interaction between Na/K-ATPase and IP3R, the LLC-PK1 cells were transfected with 2.0 μg of eYFP and αNT-eYFP constructs, respectively. After 24 h, the total cell lysates (including both the transfected and untransfected cells) were immunoprecipitated with an anti-IP3R antibody and then probed for co-precipitated α1. As depicted in Fig. 7A, expression of the N terminus caused more than 45% reduction in the co-precipitated α1 subunit. Because the transfection efficiency was about 50%, these findings clearly demonstrated that the α1 N terminus was an effective dominant negative regulator of the interaction between the Na/K-ATPase and IP3R.
Interaction of Na/K-ATPase with IP₃R

Knockdown of the Na/K-ATPase Also Inhibits Angiotensin II and EGF-induced Ca²⁺ Release—If the Na/K-ATPase is a key scaffold protein for bringing IP₃R to the proximity of plasma membrane, we would expect that α₁ knockdown should also affect other stimuli-induced ER Ca²⁺ release. To test this, we determined the effect of Na/K-ATPase knockdown on other G protein-coupled receptors as well as EGF receptor-mediated Ca²⁺ release. We have used EGF as a positive control to study ouabain-induced protein tyrosine phosphorylation in LLC-PK1 cells (25). Others have shown that angiotensin II stimulates signal transduction in LLC-PK1 cells (31). As illustrated in Fig. 8A, angiotensin II was effective in stimulating ER Ca²⁺ release in LLC-PK1 cells. Like ATP, its effect on Ca²⁺ was significantly diminished in the Na/K-ATPase knockdown PY-17 cells (Fig. 8B). Moreover, when the same experiments were repeated with EGF, we found that EGF-induced Ca²⁺ release was also attenuated (Fig. 8, C and D).

DISCUSSION

In this study we reported two major findings. First, knockdown of the Na/K-ATPase changed the subcellular distribution of IP₃R and attenuated the ER Ca²⁺ release provoked by the activation of a number of plasma membrane receptors. Second, disruption of the interaction between the Na/K-ATPase and IP₃R by overexpression of the α₁ N terminus functioned as the Na/K-ATPase knockdown and reduced ATP-induced ER Ca²⁺ release. These new findings suggest a novel structural and functional role of Na/K-ATPase, which may serve as a focal point for recruiting the ER IP₃Rs into proximity of the plasma membrane receptors and facilitating IP₃R-mediated Ca²⁺ signaling (Fig. 9).

The Na/K-ATPase Enhances the Efficiency of Signal Transmission from the Plasma Membrane Receptor Activation to the ER Ca²⁺ Release—We provided evidence that the Na/K-ATPase is required for several extracellular stimuli to efficiently stimulate ER Ca²⁺ release in LLC-PK1 cells. Specifically, both knockdown of the Na/K-ATPase and overexpression of the N terminus of the α₁ subunit were sufficient to attenuate ATP-induced Ca²⁺ signaling. Although the knockdown of Na/K-ATPase did result in an increase in intracellular Na⁺ concentration, it is unlikely that the de-sensitization of the receptor activation-provoked ER Ca²⁺ release is because of the simple inhibition of the pumping function of the Na/K-ATPase. First, although PY-17 cells and A4-11 cells exhibited similar pumping activity (3), ATP-induced ER Ca²⁺ release was further reduced in PY-17 cells that expressed less Na/K-ATPase (Fig. 2B). Second, complete inhibition of Na/K-ATPase by ouabain did not change ATP-induced ER Ca²⁺ release in LLC-PK1 cells (Fig. 2D). Finally, there was no detectable change in basal Ca²⁺ in the Na/K-ATPase knockdown cells (Fig. 2B), and the ER Ca²⁺ content was similar among different cell lines (Fig. 3F).

As shown in Figs. 3 and 5, the Na/K-ATPase knockdown specifically attenuated the IP₃-induced Ca²⁺ signaling but not DAG-provoked activation of PKCε. A similar pattern of Ca²⁺ regulation has been reported in neuronal cells exposed to agonists of muscarinic M1 and B2 bradykinin receptors (16). Specifi-
The role of Na/K-ATPase in the formation of the junctional Ca\(^{2+}\) signaling microdomain. Caveolar Na/K-ATPase interacts with the ER IP\(_3\)R through its N terminus. This interaction could be further stabilized by the cytoskeleton protein such as ankyrin (8, 41) and enhanced by ouabain-activated signaling pathways (7, 9, 10). Functionally, this interaction between Na/K-ATPase and IP\(_3\)R helps targeting IP\(_3\)R to the proximity of PLC-coupled plasma membrane receptors (e.g. GPCR, EGFR, and Na/K-ATPase), thus facilitating the IP\(_3\)-mediated Ca\(^{2+}\) release from the ER. SOCs, store-operated calcium channels; PM, plasma membrane; N, N terminus; C, C terminus; CaM, calmodulin.

FIGURE 9. The role of Na/K-ATPase in the formation of the junctional Ca\(^{2+}\) signaling microdomain. Caveolar Na/K-ATPase interacts with the ER IP\(_3\)R through its N terminus. This interaction could be further stabilized by the cytoskeleton protein such as ankyrin (8, 41) and enhanced by ouabain-activated signaling pathways (7, 9, 10). Functionally, this interaction between Na/K-ATPase and IP\(_3\)R helps targeting IP\(_3\)R to the proximity of PLC-coupled plasma membrane receptors (e.g. GPCR, EGFR, and Na/K-ATPase), thus facilitating the IP\(_3\)-mediated Ca\(^{2+}\) release from the ER. SOCs, store-operated calcium channels; PM, plasma membrane; N, N terminus; C, C terminus; CaM, calmodulin.

cally, it was found that although both receptors were equally effective at activating PKC, only the B2 receptor agonist could elicit robust ER Ca\(^{2+}\) release from the IP\(_3\)Rs. Mechanistically, this difference in receptor-mediated Ca\(^{2+}\) signaling is because of the different distribution of these receptors in the plasma membrane. Although B2 receptors and IP\(_3\)Rs are physically coupled, the M1 receptors are remote from IP\(_3\)Rs, leading to diffusion of IP\(_3\) over a significant distances and attenuation of IP\(_3\)-induced Ca\(^{2+}\) signaling. Consistent with this mode of regulation, we observed that knockdown of Na/K-ATPase shifted the ATP concentration curve to the right (Fig. 5). Needless to say, other possibilities may also be in play. For example, knockdown of Na/K-ATPase reduces the interaction between the Na/K-ATPase and IP\(_3\)R, which may simply reduce the sensitivity of IP\(_3\)R to IP\(_3\), or change the channel opening properties as suggested previously (10). These issues have to be addressed in future studies.

In addition to the reduction in ATP sensitivity, the maximal effect of ATP on Ca\(^{2+}\) release was also attenuated by Na/K-ATPase knockdown (Fig. 5). Although the exact molecular basis of this defect is unknown, it is of great interest to note the existence of a well characterized Ca\(^{2+}\)/calmodulin-mediated negative feedback mechanism in the cell (32, 33). This regulatory mechanism serves as a filter to efficiently inhibit slow rather than rapid rises in IP\(_3\). If the local IP\(_3\) does not reach a sufficient concentration with appropriate speed, the second messenger will fail to or cannot sufficiently initiate the Ca\(^{2+}\) signaling. Taken together, our data support the notion that the Na/K-ATPase plays an important role in coupling the activation of the plasma membrane receptors to the opening of ER IP\(_3\)Rs.

The Nonpumping Na/K-ATPase as a Structural Anchor for Targeting ER IP\(_3\)Rs into Junctional Microdomains—There is solid evidence that a privileged communication between the plasma membrane receptors and the opposing ER is established in many cells to ensure specificity and efficiency in Ca\(^{2+}\) signaling (16, 34, 35). Structurally, these communications occur in junctional microdomains where the plasma membrane and the opposing ER are separated by a small gap of about 10–25 nm (36). Interestingly, in neuronal cells the coupling between metabotropic glutamate receptors and ER IP\(_3\)Rs appears to be mediated by the Homer family of proteins (37).

Caveolae are uncoated invaginations of the cell surface and are highly enriched in signaling proteins, including receptor tyrosine kinases, G protein-coupled receptors, Ca\(^{2+}\)-ATPase, and several PKC isoforms (38). Direct measurements of calcium changes in endothelial cells suggest that caveolae may be sites that regulate intracellular Ca\(^{2+}\) concentration and Ca\(^{2+}\)-dependent signal transduction (39). Moreover, there is evidence that caveolae are in close contact with the ER and these contacts are mechanically stable (40). Because the nonpumping Na/K-ATPase represents an abundant caveolar protein, our new findings lead us to speculate that the nonpumping Na/K-ATPase may serve as a structural anchor for recruiting and targeting IP\(_3\)R to the caveolae/ER junctional microdomain (Fig. 9). This notion is supported by the following observations. First, overexpressing the α1 N terminus was sufficient to block the interaction between Na/K-ATPase and IP\(_3\)Rs (Fig. 7). Consequently, it also diminished ATP-induced ER Ca\(^{2+}\) release (Fig. 6). Second, there was a pool of IP\(_3\)R3 resided at the cell periphery in LLC-PK1 cells. This pool of IP\(_3\)R3 disappeared in the Na/K-ATPase knockdown cells (Fig. 4). Finally, the Na/K-ATPase is known to interact with ankyrin and spectrins (41–43). These interactions will certainly provide structural stability for the formation of junctional Ca\(^{2+}\) signaling microdomains.

It is important to note that in addition to serving as a plasma membrane anchor for targeting IP\(_3\)Rs, the nonpumping Na/K-ATPase is also associated with PLC-γ and Src family kinases in the caveolae (6, 9). It is known that Src family kinases can phosphorylate IP\(_3\)Rs and make them more sensitive to IP\(_3\) stimulation (44). Thus, it is logical for us to suggest that the formation of junctional microdomain of the Na/K-ATPase-PLC-Src-IP\(_3\)R complex ensures the efficient signal transmission from the activation of plasma membrane receptors into ER Ca\(^{2+}\) release (Fig. 9). This notion was further supported by the fact that the Na/K-ATPase knockdown not only de-sensitized ATP-, but also angiotensin II-induced as well as EGF-induced ER Ca\(^{2+}\) release.

In conclusion, our study highlights a novel role of the Na/K-ATPase in regulation of ER IP\(_3\)R-mediated Ca\(^{2+}\) mobilization. It is worth noting that the expression of Na/K-ATPase is highly regulated by physiological stimuli, and significant down-regulation occurs under many pathological conditions (45–47). Thus, changes in cellular Na/K-ATPase could have profound effects on plasma membrane receptor-initiated Ca\(^{2+}\) signaling. Moreover, de-sensitization of the receptor-mediated Ca\(^{2+}\) signaling by the down-regulation of Na/K-ATPase may lead to an increased release of the ligands via a feedback mechanism, which could result in an overstimulation of the plasma mem-
braneous receptors and then subsequent maladaptive changes in receptor function.

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