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Inhibition of Na,K-ATPase Activates PI3 Kinase and Inhibits Apoptosis in LLC-PK1 Cells

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In the present study we used LLC-PK1 cells, a porcine renal proximal tubular cell line, to investigate whether PI3 kinase activation was involved in the anti-apoptotic effect of ouabain, a specific inhibitor of Na,K-ATPase. Apoptosis was induced by actinomycin D (Act D, 5 μM) and assessed by appearance of hypodiploid nuclei and DNA fragmentation. Ouabain attenuated Act D-induced apoptotic response in a dose-dependent manner. Incubation in a low K^+ medium (0.1 mM) which is another way to decrease Na,K-ATPase activity also had anti-apoptotic effect. Both ouabain and low K^+ medium increased the PI3 kinase activity in p85 immunoprecipitates. Ouabain, as well as incubation in the low K^+ medium, also increased the phosphorylation of Akt. Inhibition of PI3 kinase by either wortmannin or LY294002 reversed the cytoprotective effect of ouabain. These data together indicate that inhibition of Na,K-ATPase activates PI3 kinase in LLC-PK1 cells which could then exert the cytoprotective effect.

Key Words: **ouabain; potassium; Na,K-ATPase; PI3 kinase; Akt; LLC-PK1 cells; actinomycin D; apoptosis.**

The phosphatidylinositol 3 kinase (PI3 kinase) is a group of ubiquitous lipid kinases that catalyzes the synthesis of 3′-phosphorylated inositides and regulates various cellular processes. Class I PI3 kinase are heterodimers, consisting of a 110-kDa catalytic subunit (p110) and a 85 or 55 kDa regulatory subunit (p85/p55). The p85 subunit contains two SH2 domains, one SH3 domain, a Bcr homology domain, and proline-rich sequences, conferring the multiple regulatory mechanisms on PI3 kinase catalytic activities. The most potent inhibitor of PI3 kinase is wortmannin, a fungal metabolite, that irreversibly inactivates the enzyme, but a novel and structurally unrelated compound, LY294002, also inhibits PI3 kinases with a great specificity, and this inhibition is reversible. Several forms of PI3 kinases have been cloned from a wide range of tissues and organisms. These enzymes show great conservation within their catalytic and lipid kinase domains. Based on their substrate specificity, PI3 kinases are divided into three major classes, and only Class I PI3 kinases phosphorylate the 3-position of the inositol ring of phosphatidylinositol 4, 5-bisphosphate [PI (4, 5) P_2] (1). The lipid product, PI (3–5) P_3 serves as a membrane anchored secondary messenger by recruiting and activating protein kinases such as Akt and certain isoforms of protein kinase C. PI3 kinase has been implicated in the regulation of cell growth and inhibition of apoptosis, and also in intracellular vesicle trafficking, secretion, and cytoskeletal organization. The inhibitory effect of PI3 kinase on cell death has received a great deal of attention recently, and the serine/threonine kinase Akt has been identified as a key enzyme that mediates the PI3 kinase-dependent anti-apoptotic effect of a variety of growth factors like insulin, IGF-1, EGF, and PDGF (2).

Following PI3 kinase activation, Akt binds to D3 phosphoinositides via its amino-terminal pleckstrin homology domain, thereby translocating to the plasma membrane, where it becomes phosphorylated at Thr^{308} and Ser^{473} and therefore activated by another PIP3-regulated kinase, PDK1. The activated Akt then acts on several downstream effectors, such as BAD, FKHRL1, IKKα and β, to exert its anti-apoptotic effect (3).

Na,K-ATPase is a membrane-bound enzyme that maintains the inward Na^+ and outward K^+ electrochemical potential gradients. Partial inhibition of Na,K-ATPase by ouabain stimulates DNA and protein

Abbreviations used: Act D, actinomycin D; $[K^+]o$, extracellular K^+ concentration; PI3 kinase, phosphatidylinositol 3 kinase.

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syntheses as well as cell replication (4, 5). Emerging evidence demonstrates that the partial inhibition of Na,K-ATPase also provides a prosurvival signal in addition to its mitogenic effect. The pretreatment of cultured cortical neurons with ouabain significantly lowers subsequent hypoxic cell death (6). Recently, the sublethal concentrations of ouabain have been found to block apoptosis induced by serum deprivation or treatment with staurosporine or okadaic acid in vascular smooth muscle (7). Similarly, low potassium medium, which inhibits Na,K-ATPase, has been shown to prevent the onset of apoptosis in cultured rat cerebellar granule cells (8). Since PI3 kinase plays a pivotal role in the inhibition of apoptosis, in the present study we used LLC-PK1 cells, a porcine renal proximal tubular cell line, to investigate whether PI3 kinase activation mediated the effect of ouabain.

MATERIALS AND METHODS

Cells and materials. The LLC-PK1 cells (American Tissue Culture Collections, Manassas, VA) were grown in Medium 199 plus 3% fetal bovine serum at 37°C with 5% CO_2. All chemicals were purchased from Sigma (St. Louis, MO) except for wortmannin and LY294002 that were purchased from Calbiochem (San Diego, CA).

Cell viability assay. The cells were plated down at 6×10^4/well (confluent) in 96-well plates and incubated for 16 to 18 h prior to treatments. After treatments, the cells were stained with 0.5% crystal violet dye in methanol for 8 to 10 min at 22°C and then washed three times with 1× PBS buffer. The absorption measured at 550 nm by a multi-well plate reader was used as an index for cell viability (9).

Fluorometric analyses of hypodiploid nuclei. After treatment until 60 to 80% of them detached from the wells, the cells (10^6) were collected and incubated in a hypotonic fluorochrome solution (Propidium iodide 0.5 mg/ml in 0.1% sodium citrate plus 0.1% Triton X-100) overnight at 4°C. The propidium iodide fluorescence of each individual nucleus was measured with excitation of 488 nm and emission of 620 nm. The cell debris and RNA were excluded from analyses by appropriately raising the forward scatter threshold. Hypodiploid nuclei due to condensation of nuclear chromatin appeared at sub G_0/G_1 position (10).

PI3 kinase activity assay. Cells were plated at 10^5 cells/well with DMEM plus 10% fetal bovine serum in a 6-well plate. The cells were switched to serum-free DMEM for an additional 48 h when they reached about 60% of confluence. Cells were then treated with ouabain or low K^+ medium at 37°C for 10 min, before lysis in 0.5 ml lysis buffer that was composed of 40 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 2 mM orthovanadate, 1 mM DTT, 10 mM EGTA, 0.01 mM PMSF, 2 μg/ml leupeptin and 20 mM Hepes (pH 7.5). PI3 kinase was immunoprecipitated with the antibody against the p85 regulatory subunit (Upstate Technology, Waltham, MA). After they were washed with lysis buffer twice, the pellets were incubated in a buffer containing 100 mM NaCl, 4 mM $MgCl_2$, 0.5 mg/ml phosphatidylinositol, 250 μM [γ-32P] ATP (20 μCi), and 20 mM Tris–HCl (pH 7.4) at 22°C for 10 min. Reactions were terminated with CHCl3:CH3OH:0.6N HCl (100:100:0.75 v/v), and the lipids were separated by thin layer chromatography. The bands corresponding to phosphatidylinositol-3-phosphate were quantitated using a PhosphoImager (11).

Detection of Phosphor-Akt. Cells were cultured and treated in the same way as in PI3 kinase activity assays. Cells were lysed in sample loading buffer containing 0.1% glycerol, 6% SDS, 0.05% 2-mercaptoethanol and 0.3 mM Tris–HCl, pH 6.8), the proteins were resolved in a 10% SDS–polyacrylamide gel followed by transfer to a PVDF membrane. The membranes were probed with an antibody against phosphorylated Akt at the position of Ser^{473} (New England Biolabs, Beverly, MA). The membranes were also probed with an antibody against Akt to control the amount of loading of samples. The enhanced chemiluminescent method was used to visualize signals (Amersham, Little Chalfont, England) (12).

DNA fragmentation assay. The fragmented DNA in cytoplasma from 2×10^6 cells/group was detected according to Sei *et al.* (13). Briefly, after treatment with Act D until 80% of the cells detached from the dishes, the cells were collected and lysed in 10 mM EDTA, 0.5% Triton X-100, and 5 mM Tris–HCl (pH 8.0), and centrifuged at 10,000*g* for 30 min. The supernatants were digested with 0.1 mg/ml proteinase K at 50°C for 60 min, extracted with phenol/chloroform, and precipitated with 2 volumes of 100% ethanol. The precipitates were treated with 0.5 mg/ml RNase. The DNA was resolved in 1.8% agarose gel.

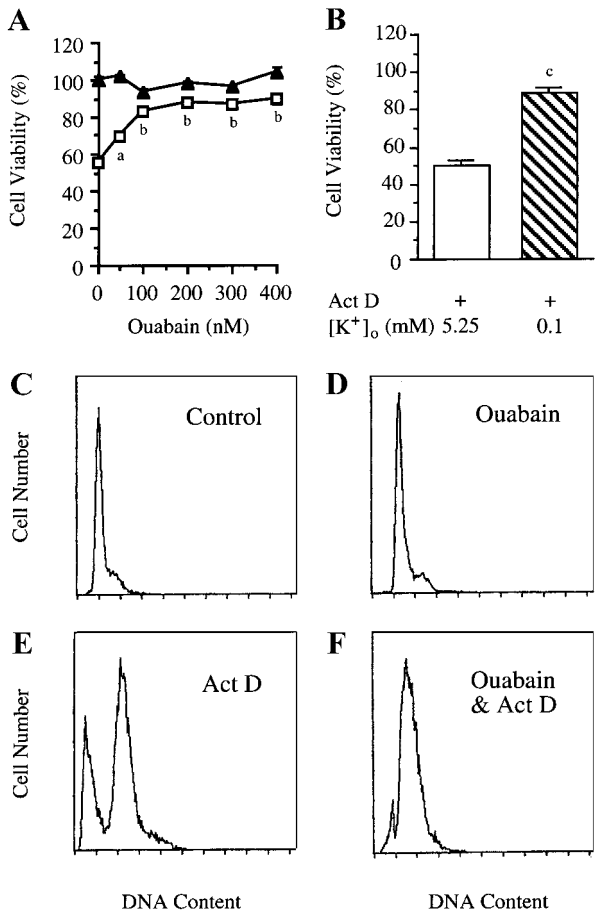

FIG. 1. Ouabain inhibited Act D (5 μM)-induced cell death in a dose-dependent manner in LLC-PK1 cells (A). The reduction of medium K^+ concentration to 0.1 mM also had a similar effect (B). Cell viability was examined 24 h after treatments by crystal violet assays. $^aP < 0.05$ (ANOVA), $^bP < 0.01$(ANOVA), and $^cP < 0.001$ as compared with Act D alone (Student's *t* test, $n = 3$). Triangle, Ouabain alone; and Square, Ouabain and Act D. Ouabain (300 nM) attenuated Act D (5 μM)-induced hypodiploid nuclei. The hypodiploid nuclei were analyzed with propidium iodide-based flow cytometry after the cells were treated until 60 to 80% detached from the dishes. Each panel is a representative of four independent experiments.

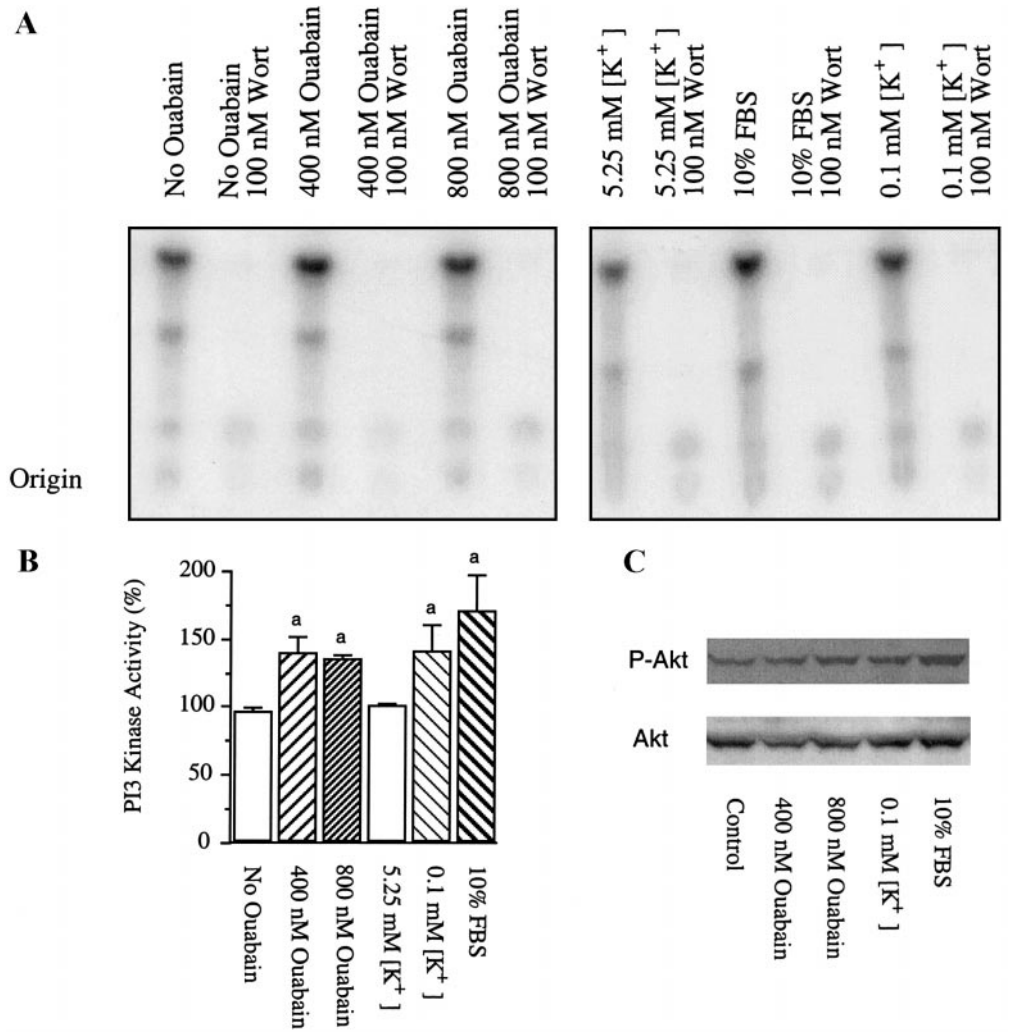

FIG. 2. Ouabain and 0.1 mM $[K^+]_o$ increased the activities of PI3 kinase (A & B) and Akt kinase (C) in LLC-PK1 cells. PI3 kinase activity was measured in p85 immunoprecipitates. $^aP < 0.05$ (ANOVA for ouabain, Student's *t* test for low $[K^+]_o$ and fetal bovine serum, $n = 5$, except for the group of 10% FBS, $n = 2$). Akt activity was determined by Western analyses. Data is a representative of four independent experiments.

Statistics. The numerical data are means of three experiments and ± standard errors. Each experiment was performed in duplicate or triplicate. Statistical analyses were performed with Student's *t* test, or one-way analysis of variance (ANOVA) as appropriate. Post multiple comparisons were made by Dunnett test (ANOVA), and $P < 0.05$ was considered a difference of statistical significance.

RESULTS

Ouabain and low extracellular K^+ concentration inhibits actinomycin D (Act D)-induced apoptosis. LLC-PK1 cells do not respond with apoptosis to serum withdrawal, therefore, Act D (5 μM) was used. As shown in Fig. 1A, increased concentrations of ouabain exerted an inhibitory effect on Act D-induced cell death based on cell viability analyses. This effect was associated with suppression of the characteristics of apoptosis such as hypodiploid nuclei and DNA fragmentation (Figs. 1C to 1F and Fig. 3C). Similar to ouabain treatment, incubation of the cells with a low potassium medium (0.1 mM), which is also known to reduce Na,K-ATPase activity, also resulted in inhibition of Act D-induced cell death (Fig. 1B).

Ouabain and low K^+ medium increases PI3 kinase activity and phosphorylation of the Akt kinase. To determine whether PI3 kinase was involved in the cytoprotection of ouabain and low K^+ medium, the effects of ouabain and low K^+ medium on the activity of the kinase was examined in p85 immunoprecipitates following treatments. In order to maximize the activation of PI3 kinase in a short period of time, the concentrations of ouabain were increased to 400 and 800 nM. As shown in Figs. 2A and 2B, ouabain (800 nM) and low K^+ medium (0.1 mM) increased PI3 kinase activity by 41 and 40%, respectively. While this increase was moderate, it is noted that the treatment of the cells

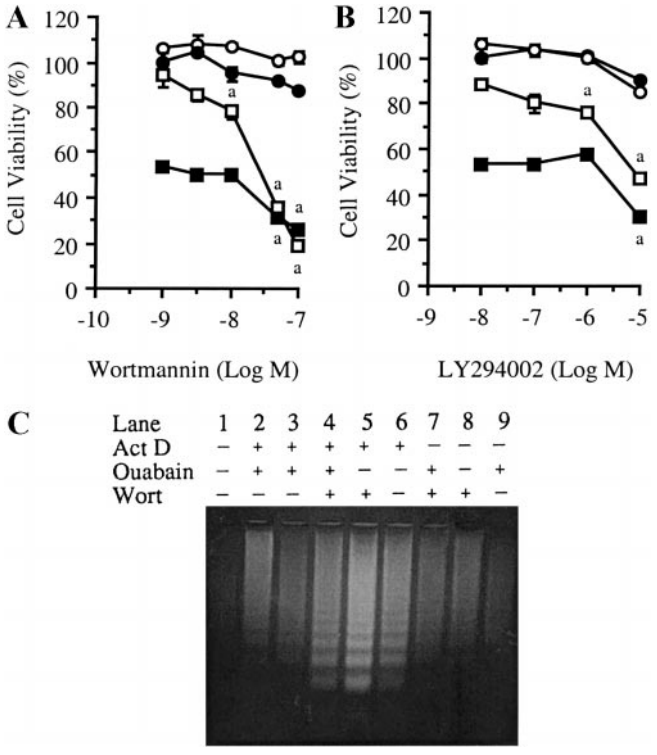

FIG. 3. Wortmannin (A) or LY294002 (B) inhibited the cytoprotection of ouabain (300 nM) in a dose-dependent manner in LLC-PK1 cells. Open circle, Wortmannin or LY294002 alone; solid circle, Wortmannin or LY294002 plus ouabain; open square. Wortmannin or LY294002 plus Act D and ouabain; solid square, Wortmannin or LY294002 plus Act D. Cell viability was examined by crystal violet assays 24 h after treatments. $^{a}P < 0.01$ as compared with no wortmannin or LY294002 (ANOVA, $n = 3$). Wortmannin (50 nM) abolished the effect of ouabain on Act D (5 μM)-induced DNA fragmentation. Lane 1, control; Lane 2, ouabain 100 nM and Act D 5 μM; Lane 3, ouabain 300 nM and Act D 5 μM; Lane 4, ouabain 300 nM, Act D 5 μM and wortmannin (Wort) 50 nM; Lane 5, Act D 5 μM and wortmannin (Wort) 50 nM; Lane 6, Act D 5 μM; Lane 7, ouabain 300 nM and wortmannin (Wort) 50 nM; Lane 8, wortmannin (Wort) 50 nM; and Lane 9, ouabain 300 nM. The DNA in the cytoplasma was extracted and resolved in 1.8% agarose gel.

with 10% fetal bovine serum also increased the activity of the enzyme only by 74% (Figs. 2A and 2B).

Since Akt kinase is one of the major targets of PI3 kinase, Western analyses were used to examine whether ouabain and low K^+ medium also increased Akt phosphorylation at the position of Ser^{473}. As shown in Fig. 2C, both ouabain and low K^+ medium increased the amount of phosphor-Akt. It is important to note that Akt was already partially phosphorylated in control cells.

Inhibition of PI3 kinase reverses the effect of ouabain and low K^+ medium on apoptosis. To further determine if PI3 kinase was involved in the cytoprotective effect of ouabain on Act D-induced cell death, the effect of ouabain was examined in the presence of wortmannin or LY294002. As shown in Fig. 3, both wortmannin and LY294002 inhibited the effect of ouabain in a dose-dependent manner. Wortmannin at 50 nM or LY294002 at 10 μM completely abolished the effect of ouabain. Wortmannin or LY294002 also augmented Act D-induced apoptosis, possibly resulting from the inhibition of a high basal PI3 kinase activity. However, wortmannin or LY294002 alone or in combination with 300 nM ouabain had no significant effect on cell viability (Figs. 3A and 3B).

DISCUSSION:

The present study has shown that the partial inhibition of Na,K-ATPase by treatment with ouabain and incubation in a low K^+ medium resulted in the activation of PI3 kinase and Akt, which could mediate the cytoprotective action of these two manipulations. Partial inhibition of Na,K-ATPase also increases the activity of the pump. Compatible with the present observation, Aydemir-Koksoy and Allen have recently demonstrated that LY 294002 inhibits the upregulation of Na,K-ATPase binding sites by low K^+ in vascular smooth muscle cells (14). The mechanism by which Na,K-ATPase inhibition leads to PI3 kinase activation is not clear at present time. However, inhibition of the pump leads to a decrease in membrane potential (15) and is associated with elevated intracellular Na levels (7) and increased cell volume (16). All these effects have been reported to increase PI3 kinase activity. For example, depolarization of membrane potential has been shown to activate PI3 kinase in sympathetic neurons (17). Increased intracellular Na^+ concentration is able to stimulate PI3 kinase activity in the murine kidney inner medullary cells (18), and increased cell volume induced by hypoosmolar swelling also activates PI3 kinase in cardiac myocytes (19).

In LLC-PK1 cells, ouabain and low K^+ medium did not increase the activity of PI3 kinase in p85 immunoprecipitates more than 40%, which is less than usually observed in other experimental systems with other stimuli (11). However, we also noted that fetal bovine serum at the concentration of 3% failed to stimulate the enzyme (data not shown) and only increased the activity of the enzyme by 74% when its concentration was raised to 10% (Fig. 2B). This, together with the presence of phosphorylated Akt in serum-deprived cells suggests that there is already elevated PI3 kinase activity and partial activation of Akt in the quiescent cells, which may explain why these cells did not display apoptosis after serum withdrawal.

Although the functions and regulations of PI3 kinase in renal proximal tubules remain largely unknown, available data suggest that PI3 kinase is involved in the processes described in other systems. For example, PI3 kinase mediates the mitogenic effects of albumin (20) and lysophosphatidic acid (21), and survival effect of hepatocyte growth factor (22). PI3 kinase has been

also shown to regulate endocytosis of albumin (23) and Na,K-ATPase upon exposure to dopamine (24).

The present observations might be relevant to cardiovascular regulatory mechanisms, especially under pathological conditions. The human circulation contains four seemingly endogenous and readily distinguishable inhibitors of Na,K-ATPase. Of these, one has been shown to be a novel isomer of ouabain in which the location and orientation of two or more steroidal hydroxyl groups differ. Among Caucasians with essential hypertension, 30–45% have elevated levels of endogenous ouabain-like compounds (25). Endogenous ouabain-like compound induces rat mesangial cell proliferation (26, 27) and hypertrophy of patient left ventricle (28). Ouabain enhances the mitogenic effect of serum in cultured vascular smooth muscle cells (29) and causes hypertrophic growth in cultured cardiac cells (30). Similar to the effect of ouabain on the cardiovascular system, potassium-deficient diets induce hyperplasia and hypertrophy in rat kidney (31, 32). Tsao *et al.* have recently demonstrated that the mitogenic effect of potassium depletion may be due to, at least in part, the increase in the local levels of IGF-1 and IGF-1 binding protein (33). The present data raise the possibility that activation of PI3 kinase and antiapoptosis plays a role in the effects of ouabain and potassium depletion *in vivo.*

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