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Association of PI3K-Akt signaling pathway with digitalis-induced hypertrophy of cardiac myocytes

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Liu L, Zhao X, Pierre SV, Askari A. Association of PI3K-Akt signaling pathway with digitalis-induced hypertrophy of cardiac myocytes. *Am J Physiol Cell Physiol* 293: C1489–C1497, 2007. First published August 29, 2007; doi:10.1152/ajpcell.00158.2007.—Our previous studies on cardiac myocytes showed that positive inotropic concentrations of the digitalis drug ouabain activated signaling pathways linked to Na^+ - K^+ -ATPase through Src and epidermal growth factor receptor (EGFR) and led to myocyte hypertrophy. In view of the known involvement of phosphatidylinositol 3-kinase (PI3K)-Akt pathways in cardiac hypertrophy, the aim of the present study was to determine whether these pathways are also linked to cardiac Na^+ - K^+ -ATPase and, if so, to assess their role in ouabain-induced myocyte growth. In a dose- and time-dependent manner, ouabain activated Akt and phosphorylation of its substrates mammalian target of rapamycin and glycogen synthase kinase in neonatal rat cardiac myocytes. Akt activation by ouabain was sensitive to PI3K inhibitors and was also noted in adult myocytes and isolated hearts. Ouabain caused a transient increase of phosphatidylinositol 3,4,5-trisphosphate content of neonatal myocytes, activated class IA, but not class IB, PI3K, and increased coimmunoprecipitation of the α -subunit of Na^+ - K^+ -ATPase with the p85 subunit of class IA PI3K. Ouabain-induced activation of ERK1/2 was prevented by Src, EGFR, and MEK inhibitors, but not by PI3K inhibitors. Activation of Akt by ouabain, however, was sensitive to inhibitors of PI3K and Src, but not to inhibitors of EGFR and MEK. Similarly, ouabain-induced myocyte hypertrophy was prevented by PI3K and Src inhibitors, but not by an EGFR inhibitor. These findings 1) establish the linkage of the class IA PI3K-Akt pathway to Na^+ - K^+ -ATPase and the essential role of this linkage to ouabain-induced myocyte hypertrophy and 2) suggest cross talk between these PI3K-Akt pathways and the signaling cascades previously identified to be associated with cardiac Na^+ - K^+ -ATPase.

cardiac glycosides; growth factor receptor; heart failure; Na^+ - K^+ -ATPase; sodium pump

SODIUM-POTASSIUM-ADENOSINETRIPHOSPHATASE catalyzes the linked active transport of Na^+ and K^+ across the plasma membranes of most mammalian cells, and this pumping function of the enzyme is specifically inhibited by ouabain and related digitalis drugs (50). In the heart, digitalis concentrations that partially inhibit Na^+ - K^+ -ATPase to cause a modest increase in intracellular Na^+ are sufficient to affect the robust cardiac Na^+ / Ca^{2+} exchanger, leading to significant increases in intracellular Ca^{2+} and cardiac contractility (5, 48, 51). This positive inotropic action of digitalis drugs has been considered to be a basis of the major classical use of these drugs for the treatment of heart failure (23, 51).

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Peng et al. (43) were the first to note that positive inotropic, but nontoxic, concentrations of ouabain also caused transcriptional regulation of several cardiac growth-related genes and hypertrophic growth of the myocytes. Extensive subsequent studies on cardiac myocytes and isolated heart preparations have indicated that these newly appreciated effects of digitalis drugs are the consequences of the drug-induced interactions of Na^+ - K^+ -ATPase subunits with neighboring membrane proteins such as Src, epidermal growth factor receptor (EGFR), and caveolins, leading to activation of multiple signal transduction pathways that link the initial cell membrane events to intracellular organelles and the nucleus (31, 37, 56). Related studies have shown that digitalis-induced signaling through Na^+ - K^+ -ATPase also occurs in a variety of cell types other than cardiac myocytes but that the downstream signaling pathways and functional consequences are clearly cell specific (1, 25, 29), emphasizing the need for the characterization of the details of digitalis-induced signaling in each cell type that may be exposed to these drugs in the course of therapy.

The involvement of phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways in the control of physiological and pathological cardiac hypertrophy is well established and under extensive study (9, 12, 33–35, 40, 49). The possibility of digitalis drug effects on this pathway in cardiac myocytes has not been studied, despite the known hypertrophic effects of these drugs on isolated cardiac myocytes (19, 43) and the fact that the hypertrophied failing heart is the main target of digitalis therapy (23, 51). Therefore, the aims of the present studies were to determine whether ouabain, the prototypic digitalis drug, affects PI3K-Akt pathways in cardiac myocytes and to begin the clarification of the relation of any such effects to other cardiac signaling pathways that are known to be regulated by digitalis drugs and to digitalis-induced hypertrophy.

MATERIALS AND METHODS

Materials. Chemicals of the highest purity and culture media were purchased from Sigma (St. Louis, MO). Antibodies against phosphorylated (Ser⁴⁷³) Akt, Akt, phosphorylated (Ser²⁴⁴⁸) mammalian target of rapamycin (mTOR), and mTOR were purchased from Cell Signaling Technology (Danvers, MA) and PI3K p110 α , PI3K p110 γ , phosphorylated JNK, phosphorylated p38, p38, phosphorylated (Ser⁹) glycogen synthase kinase (GSK)-3 β , goat anti-mouse IgG-horseradish peroxidase, and goat anti-rabbit IgG-horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA). The Akt kinase assay kit was purchased from Cell Signaling Technology, phosphatidylinositol 3,4,5-phosphate (PIP3) mass ELISA from Echelon Biosciences (Salt

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Lake City, UT), protein A-agarose and anti-PI3K p85 antibody from Upstate Biotechnology (Lake Placid, NY), LY-294002 and wortmannin from Cell Signaling Technology, and AG-1478, PD-158780, PD-98059, and PP2 from Calbiochem (San Diego, CA). All research on rats was done according to procedures and guidelines approved by the Institutional Animal Care and Use Committee.

Cell preparation and culture. Primary cultures of neonatal rat cardiac myocytes were prepared as described previously with minor modifications (43). Myocytes were dispersed from ventricles of 1- to 2-day-old Sprague-Dawley rats by digestion with 0.04% collagenase II (Worthington) and 0.05% pancreatin (Sigma) at 37°C. Noncardiomyocytes were eliminated by preplating for 1.5 h at 37°C. Myocytes were plated at a density of 8×10^2 cells/mm² in 100-mm Corning cell culture dishes in Dulbecco's modified Eagle's medium-M199 (4:1) containing 10% (vol/vol) fetal bovine serum (24 h, 37°C) and then incubated in serum-free medium for 48 h before experimentation.

Ca²⁺-tolerant adult rat cardiac myocytes were prepared from isolated hearts as described elsewhere (30, 31), and isolated adult rat Langendorff-perfused hearts were set up and used as indicated previously (41).

PIP3 and PI3K assays. The amount of PIP3 was measured by the PIP3 mass ELISA kit according to the manufacturer's instructions with use of a purified anti-PIP3 monoclonal antibody (Z-P345b).

PI3K assay was conducted as follows. After treatment, neonatal rat cardiac myocytes were lysed with 400 μ l of ice-cold buffer containing 140 mM NaCl, 10 mM HEPES, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM CaCl₂, 1 mM MgCl₂, 2 mM Na₃VO₄, 10% glycerol, 1% Nonidet P-40, 10 μ g/ml aprotinin, 50 μ M leupeptin, and 2 mM PMSF (pH 8.1) and solubilized by continuous stirring for 1 h at 4°C. After centrifugation (16,000 g, 15 min), the supernatant was collected, and 1 mg of protein (in 500 μ l) was incubated with an anti-PI3K p85 α or an anti-PI3K p110 γ antibody. After overnight incubation, protein A-agarose was added, and the immune complex was washed four times with buffer (100 mM NaCl, 1 mM Na₃VO₄, and 20 mM HEPES, pH 7.5) and resuspended in 40 μ l of buffer (180 mM NaCl-20 mM HEPES, pH 7.5). PI3K activity in the immunoprecipitates (equal amounts of precipitates as assessed by Western blotting) was assayed directly on the beads by a standard procedure as previously described (27, 58), with phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) and [γ -³²P]ATP used as substrates. The reactions were performed at room temperature and stopped after 10 min by addition of 80 μ l of 1 M HCl. The lipids were extracted with 160 μ l of chloroform-methanol (1:1), spotted on a thin-layer chromatography plate, and separated with chloroform-acetone-methanol-glacial acetic acid-H₂O (40:15:13:12:8). The radioactivity of the phosphorylated lipid products was quantified by a PhosphorImager (Molecular Dynamics).

Akt kinase assay. Akt kinase in neonatal cardiac myocytes was assayed using an Akt kinase assay kit (Cell Signaling) according to the manufacturer's instructions. Briefly, Akt was immunoprecipitated from lysates with use of an immobilized anti-Akt monoclonal antibody and assayed using a GSK-fusion protein as substrate, and the phosphorylated product was detected by Western blotting.

Measurement of hypertrophic growth in cultured cardiac myocytes. Cultured neonatal cardiac myocytes were plated in six-well plates. After 48 h of serum starvation, myocytes were treated with ouabain with or without preincubation with different inhibitors for 30 min. After 48 h, cells were trypsinized and counted and cell size was measured in a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA). Total protein was measured by Bio-Rad DC protein assay. DNA content was measured with a Quant-iT DNA broad-range assay kit (catalog no. Q33130, Molecular Probes, Eugene, OR).

Fluorescence microscopy. Staining and confocal microscopy were performed as described elsewhere with little modification (30, 31). Briefly, after treatment, myocytes were fixed with 2% paraformaldehyde for 10 min at room temperature and then permeabilized in 0.3% Triton X-100 for 10 min. Cells were blocked by Image-iTTM FX

signal enhancer (Molecular Probes) for 30 min and incubated with Alexa 488-conjugated phalloidin (Molecular Probes) for 2 h at room temperature. The coverslips were mounted with ProLong Gold anti-fade reagent (Molecular Probes). The confocal image was captured by a spectral confocal scanner (model TCS SP2, Leica, Mannheim, Germany) and microscope (model DMIRE2, Leica) equipped with a $\times 63$ oil immersion objective. Leica confocal microscope system software was used for visualization and analysis.

Immunoblot and immunoprecipitation. Immunoblot and immunoprecipitation were done as described elsewhere (30, 31).

Analysis of data. Values are means \pm SE of the results of a minimum of three experiments. Student's *t*-test was used, and significance was accepted at $P < 0.05$.

RESULTS

In the following experiments on rat cardiac myocytes, the ouabain concentrations (10–100 μ M) used for the indicated exposure durations have been shown previously not to affect myocyte viability (43), to cause small or no changes in intracellular Na⁺ concentrations, but to induce significant increases in intracellular Ca²⁺ concentrations sufficient to increase contractility and regulate the transcriptions of the growth-related genes (36, 43, 54, 56).

Ouabain has been shown to induce hypertrophy in neonatal rat cardiac myocytes (19, 43). In view of the established involvement of Akt in the regulation of cardiac hypertrophy and cardiac myocyte size (12, 40, 52), we examined the effects of exposure of neonatal myocytes to ouabain on Akt. Dose- and time-dependent activating effects of ouabain on Akt, as measured by increased Akt phosphorylation at Ser⁴⁷³, are summarized in Fig. 1. Similar ouabain-induced activations of Akt were also noted in limited experiments on isolated adult cardiac myocytes or isolated Langendorff-perfused hearts (not shown). To ensure that Akt phosphorylation was indeed indicative of Akt activation, Akt was immunoprecipitated from control and ouabain-treated neonatal myocytes, and the level of active Akt was assayed by addition of a well-established substrate of Akt, i.e., GSK-3. The kinase activity of Akt isolated from the ouabain-treated cells clearly exceeded that of the control cells (Fig. 2A). Time-dependent and ouabain-induced increases in phosphorylations of two endogenous substrates of Akt (GSK-3 and mTOR), which are known to regulate protein synthesis, are shown in Fig. 2B (8, 40). The data of Figs. 1 and 2 are consistent with a role of Akt activation in ouabain-induced hypertrophy.

Akt may be activated by PI3K-dependent or -independent pathways (8, 46). As shown in Fig. 2A, ouabain-induced activation of Akt was prevented by the established inhibitors of PI3K, i.e., wortmannin and LY-294002. Direct evidence for ouabain-induced activation of PI3K is shown in Fig. 3A by the rapid and transient increase of total PIP3 content of neonatal myocytes on exposure to ouabain. PI3K activation by ouabain was also confirmed when class IA PI3K was immunoprecipitated from control and ouabain-treated myocytes and assayed for activity by a standard procedure, with phosphatidylinositol and ATP used as substrates (Fig. 3B). When class IB PI3K was similarly immunoprecipitated with a specific antibody and assayed by the same standard procedure, there was no ouabain stimulation of this activity (Fig. 3C).

The regulatory subunit of class IA PI3K (p85 α) has been suggested to interact with the α -subunit of Na⁺-K⁺-ATPase (58). To explore the possible involvement of this protein-

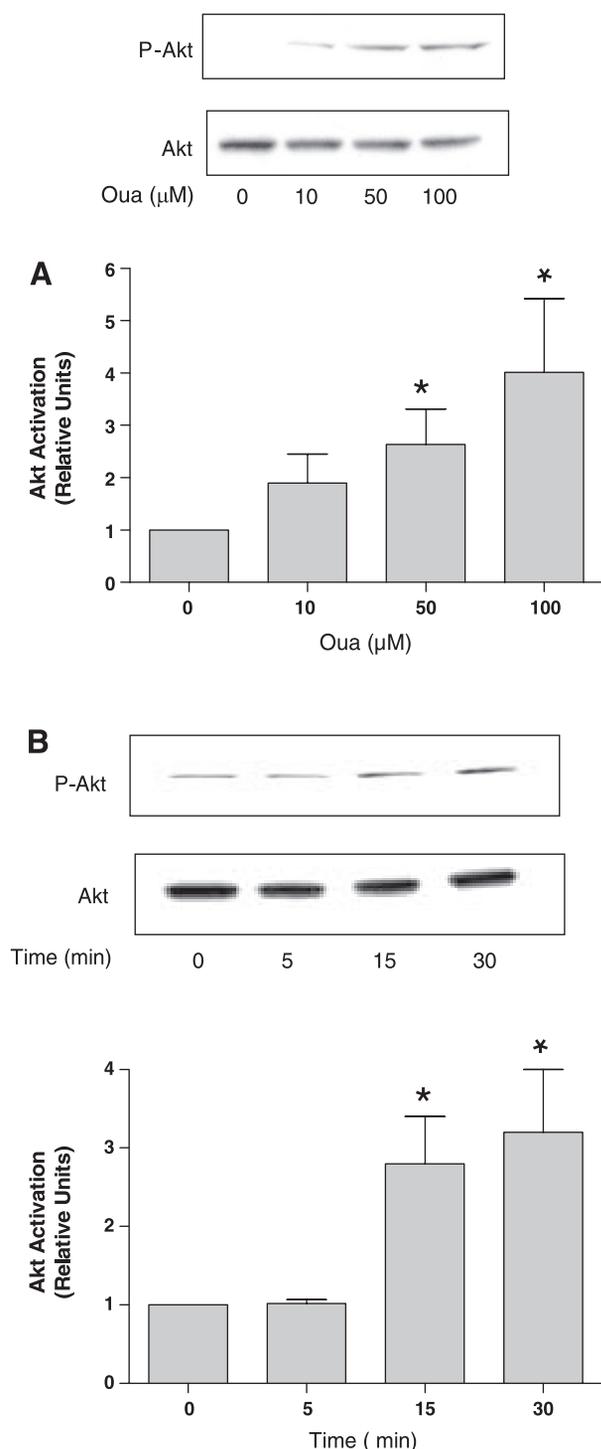


Fig. 1. Activation of Akt by ouabain in neonatal rat cardiomyocytes. *A*: myocytes were exposed to 0–100 μ M ouabain (Oua) for 15 min, and lysates were assayed for total Akt and phosphorylated (Ser⁴⁷³) Akt (p-Akt). *B*: neonatal myocytes were exposed to 100 μ M ouabain for 0–30 min and assayed as described in *A*. Activation was quantified as ratio of phosphorylated to total Akt. ($n = 4$). * $P < 0.05$ vs. control.

protein interaction in ouabain-induced activation of PI3K in cardiac myocytes, we immunoprecipitated p85 α from the lysates of control and ouabain-treated myocytes and assayed for the α_1 -subunit of Na⁺-K⁺-ATPase, since this isoform is predominant in cardiac myocytes and is responsible for most, if

not all, of ouabain-induced signaling in these cells (see DISCUSSION). The coimmunoprecipitation of the α_1 -subunit was increased significantly in response to ouabain (Fig. 4). Taken together, Figs. 1–4 clearly establish ouabain-induced activation of the PI3K-Akt pathway in cardiac myocytes through class IA PI3K, which is known to be involved in physiological cardiac hypertrophy (40).

Because in some cell types other than cardiac myocytes ouabain is known to stimulate the stress-activated kinases JNK and p38 (25, 56) and because these kinases have been implicated in maladaptive, but PI3K-dependent, growth (35, 40), we examined the effects of exposure (5–120 min) of neonatal myocytes to 100 μ M ouabain on JNK and p38 by procedures described previously (25). Neither was activated by ouabain (data not shown). The lack of effect of ouabain on these stress kinases in myocytes strengthens the suggestion that ouabain-induced hypertrophy through class IA PI3K is more akin to physiological hypertrophy.

Class IA PI3K may be activated by stimulus-induced recruitment to tyrosine kinase growth factor receptors (8, 40, 55). Since we have established that, in cardiac myocytes, ouabain transactivates Src-EGFR and the associated Ras-Raf-MEK-ERK cascade (56), we explored the relation of this pathway to the PI3K-Akt pathway by comparing the effects of several selective inhibitors on ouabain-induced activation of ERK1/2 and Akt in neonatal cardiac myocytes. Ouabain activation of ERK1/2 was blocked by inhibitors of Src (PP2), EGFR (AG-1478 and PD-158780), and MEK (PD-98059), as expected, but not by wortmannin (Fig. 5). On the other hand, ouabain-induced activation of Akt was not prevented by EGFR and MEK inhibitors but was blocked by the Src inhibitor and wortmannin (Fig. 6). Taken together, these findings suggest

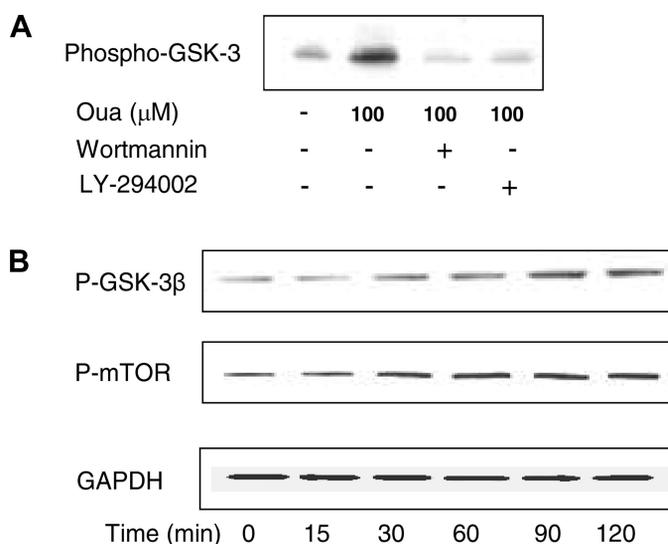


Fig. 2. *A*: ouabain-induced kinase activity of Akt in neonatal myocytes. Cells were exposed to 100 μ M ouabain in the absence or presence of 0.1 μ M wortmannin and 2 μ M LY-294002, Akt was immunoprecipitated from lysates with immobilized anti-Akt antibody, and lysate was assayed using a glycogen synthase kinase (GSK)-3 fusion protein as substrate. *B*: time-dependent increases in phosphorylations of endogenous GSK and mammalian target of rapamycin (mTOR) in neonatal myocytes by 100 μ M ouabain. Western blots were done using antibodies to phosphorylated (Ser⁹) GSK-3 β (p-GSK-3 β) and phosphorylated (Ser²⁴⁴⁸) mTOR (p-mTOR). Blots represent results of duplicate experiments.

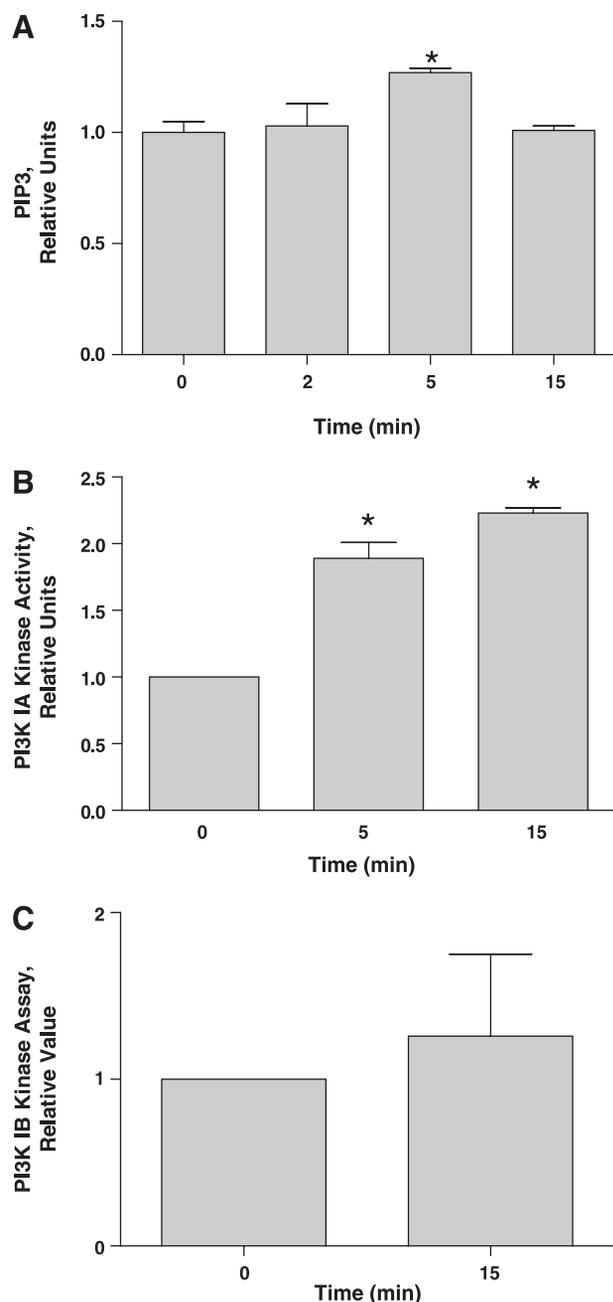


Fig. 3. Effects of 100 μ M ouabain on phosphatidylinositol 3-kinase (PI3K) in neonatal cardiac myocytes. *A*: myocytes were exposed to ouabain for 0–15 min, and lipids were extracted and immunoassayed for phosphatidylinositol 3,4,5-trisphosphate ($n = 3$). * $P < 0.05$ vs. control. *B*: myocytes were exposed to ouabain for 0–15 min, and lysates were immunoprecipitated with antibody to p85 regulatory subunit of class IA PI3K and assayed for kinase activity ($n = 3$). * $P < 0.05$ vs. control. *C*: myocytes were exposed to ouabain for 0–15 min, and lysates were immunoprecipitated with antibody to p110 γ subunit of class IB PI3K and assayed for kinase activity ($n = 5$). $P = 0.80$ vs. control.

that ouabain binding to Na⁺-K⁺-ATPase initiates activation of two pathways: the previously characterized Src-EGFR-Ras-ERK cascade and an Src-dependent PI3K-Akt pathway (Fig. 7).

To assess the relation of the above-described pathways to ouabain-induced myocyte hypertrophy, we examined the effects of pathway inhibitors on ouabain-induced changes in neonatal myocyte size and protein-to-DNA ratio. Ouabain-

induced hypertrophy (Fig. 8) was prevented by the inhibitors of PI3K and Src, but not by the inhibitor of EGFR (Fig. 9).

DISCUSSION

Activation of PI3K and/or Akt by ouabain has been reported previously in epithelial and endothelial cells in relation to ouabain-induced internalization of Na⁺-K⁺-ATPase or to stimulation by ouabain of proliferation and survival of these cells (15, 22, 27, 59). To our knowledge, the present report is the first on the activation of the PI3K-Akt pathway by a digitalis drug in cardiac myocytes, nearly all of which are unable to proliferate. Our findings clearly not only show the activation of this pathway by ouabain, but they also show the necessity of this activation to ouabain-induced hypertrophic growth of the terminally differentiated cardiac myocyte. Before the specific implications of these findings are discussed, it is necessary that we discuss a special feature of the experimental model used in the present studies, i.e., the digitalis sensitivity of the rat cardiac myocyte.

Validity of the model. The insensitivity of the rat heart compared with that of other experimental animals to the positive inotropic actions of ouabain and related drugs was known before the discovery of Na⁺-K⁺-ATPase and was shown to be accompanied by the relative insensitivity of rat heart Na⁺-K⁺-ATPase activity to ouabain soon after the discovery of this enzyme (45). What is known about the ouabain sensitivity of the signaling functions of the rat cardiac Na⁺-K⁺-ATPase? Our extensive previous studies on rat cardiac myocytes and the isolated rat heart have shown that the signaling effects of ouabain are obtained within the range of drug concentrations that cause partial inhibition of the pumping function of Na⁺-K⁺-ATPase and the resulting increase in contractility (36, 43, 54, 56). We also showed previously that

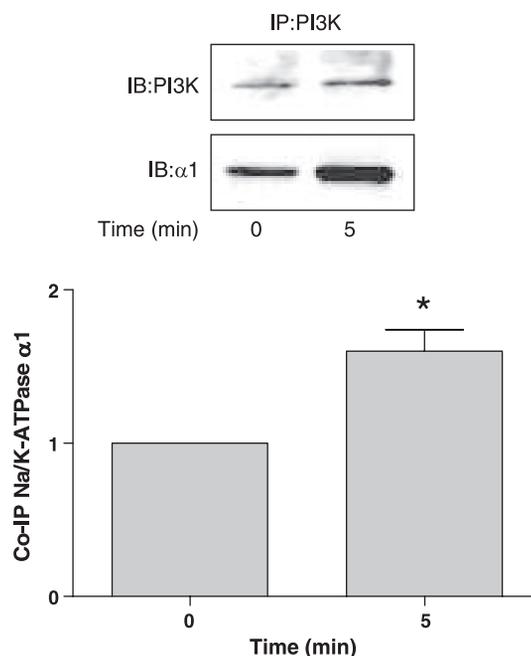


Fig. 4. Ouabain-induced increase in coimmunoprecipitation (Co-IP) of Na⁺-K⁺-ATPase α ₁-subunit with p85 subunit of class IA PI3K. Neonatal myocytes were exposed to 100 μ M ouabain for 5 min, and lysates were immunoassayed with indicated antibodies ($n = 3$). * $P < 0.05$ vs. control.

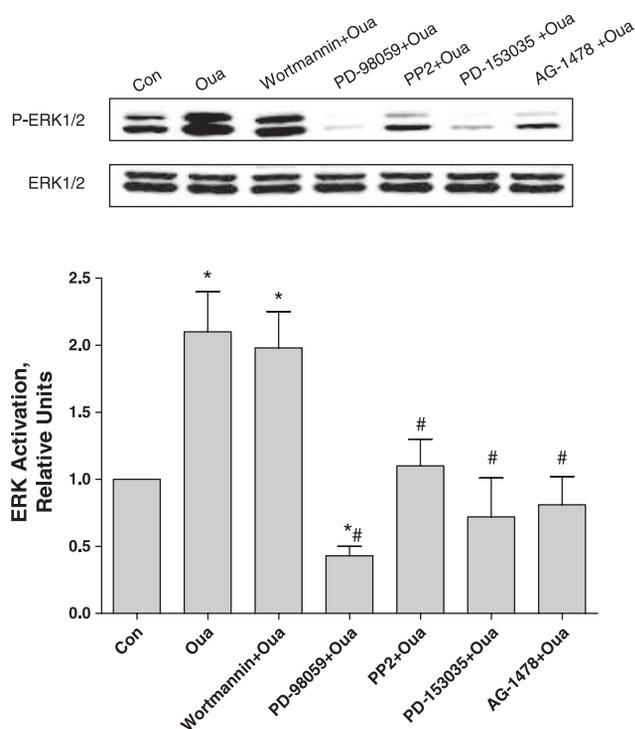


Fig. 5. Effects of inhibitors on ouabain-induced activation of ERK1/2. Neonatal myocytes were exposed to 100 μ M ouabain for 15 min in the absence and presence of inhibitors of PI3K (0.1 μ M wortmannin), MEK (30 μ M PD-98059), Src (2 μ M PP2), and epidermal growth factor receptor (1 μ M PD-153035 and 250 nM AG-1478). A: representative blots. B: quantitative comparisons of blots from 4 experiments. * P < 0.05 vs. control (Con). # P < 0.05 vs. Oua.

some of the upstream segments of the ouabain-activated pathways in myocytes are independent of the increases in intracellular concentrations of Na^+ and Ca^{2+} , which are the known consequences of inhibition of cardiac Na^+ - K^+ -ATPase (28). Although these latter findings are important to the clarification of the interrelations of the multiple ouabain-activated pathways (Fig. 7), they should not be misinterpreted to mean that ouabain-induced signaling in cardiac myocytes occurs without inhibition of Na^+ - K^+ -ATPase. All available evidence indicates that, in rat cardiac myocytes, the pumping and the signaling pools of the enzyme are the same (30), that ouabain-induced partial inhibition of the enzyme and the resulting positive inotropy go hand-in-hand (Fig. 7), and that the signaling is being done by the inhibited enzyme (30, 31, 56). Therefore, the following legitimate question may be raised: Since the ouabain concentrations (\sim 5–100 μ M) that induce signaling, positive inotropy, and pump inhibition in rat cardiac myocytes and the rat heart are two to three orders of magnitude larger than the effective blood levels of digitalis drugs in patients with heart failure (50) or any physiological levels of the postulated digitalis-like hormones (47), is any pharmacological or physiological relevance associated with studies of the effects of such large ouabain concentrations on an unusually insensitive model? The answer is yes, because the following characteristics of rat and mouse cardiac Na^+ - K^+ -ATPases are well established: 1) In these rodent myocytes, the house-keeping α_1 -isoform of the enzyme constitutes \sim 80–90% of the total cellular content, and the remainder minor component is the α_2 -isoform in the adult or the α_3 -isoform in the neonatal

myocyte (7, 14, 32). 2) These rodent α_1 -isoforms, with ouabain K_i values in the micromolar range, are about two to three orders of magnitude less sensitive than the rat and mouse α_2 - and α_3 -isoforms and various human isoforms, all of which have ouabain K_i values in nanomolar range (7, 32). 3) Although the positive inotropic action of ouabain on rat or mouse hearts or myocytes may be exerted through either isoform, \sim 80–90% of the maximal inotropy is through the predominant, but relatively insensitive, α_1 -isoform. This was established long ago (48) and has been confirmed by more recent elegant studies (13, 14). 4) The micromolar dose ranges of ouabain that produce signaling in rat cardiac myocytes (Fig. 1) (24, 28, 36) clearly indicate that, as in the case of positive inotropy, most of the signaling effects are through the α_1 -isoform, although minor contributions of the α_2 - and α_3 -isoforms have not been ruled out. For these reasons, we suggest that the isolated rat heart or rat cardiac myocytes are valid models for the study of ouabain-induced signaling, with the reasonable expectation that, in cardiac preparations that are naturally more ouabain sensitive, the findings would be similar to those in the model, but at drug concentrations lower by about two to three orders of magnitude. This expectation has been supported by our studies comparing ouabain-induced signaling in rat and guinea pig isolated hearts (37), and its more rigorous testing will be possible with the recent availability of transgenic “human-like” mice, in which the predominant ouabain-insensitive α_1 -isoform has been converted to a highly sensitive enzyme (14).

Ouabain-induced hypertrophy and activation of PI3K. Of the multiple forms of PI3K, those of the heterodimeric class I (IA and IB) have been studied most extensively (8, 40, 55). In the heart and isolated cardiac myocytes, it is well established that class IA PI3K, through the activation of Akt and its downstream targets, regulates the physiological increase in cell size and cardiac hypertrophy, such as that induced by insulin-

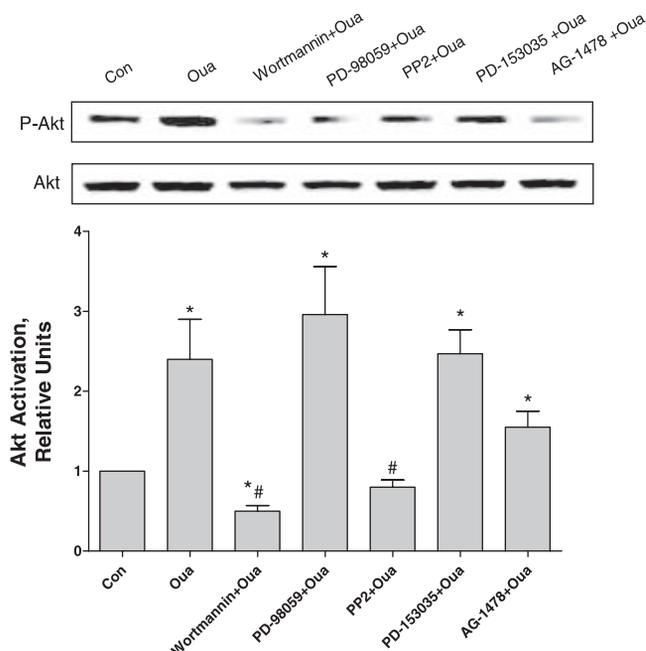
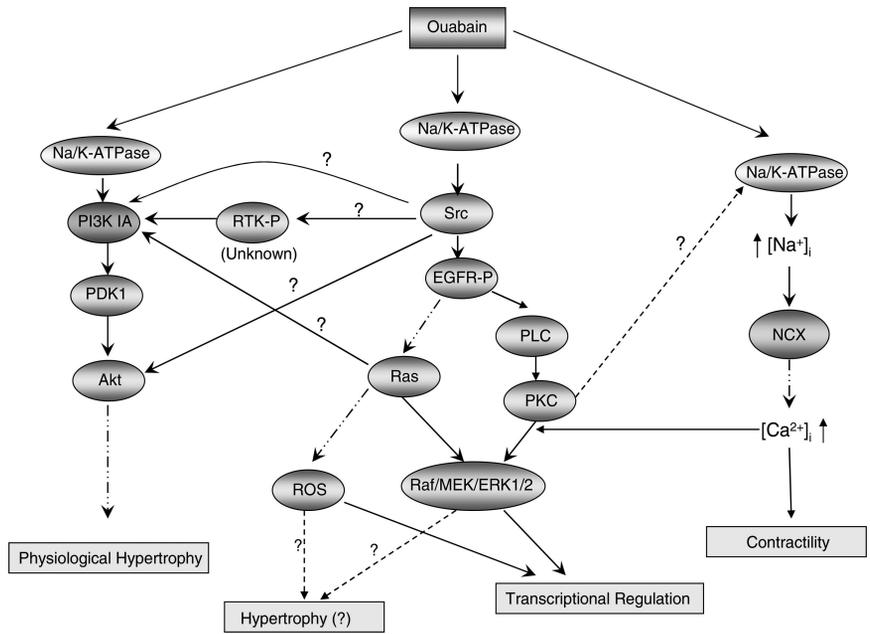


Fig. 6. Effects of various inhibitors on ouabain-induced activation of Akt. Experiments were done as described in Fig. 5 legend (n = 4). * P < 0.05 vs. control. # P < 0.05 vs. Oua.

Fig. 7. Ouabain-induced positive inotropy, the 2 parallel ouabain-activated signaling pathways that are linked to cardiac myocyte Na⁺-K⁺-ATPase, and their postulated interrelations. RTK-P, phosphorylated receptor tyrosine kinase; EGFR-P, phosphorylated epidermal growth factor receptor; PDK1, phosphoinositide-dependent kinase-1; ROS, reactive oxygen species; NCX, Na⁺/Ca²⁺ exchange; [Na⁺]_i and [Ca²⁺]_i, intracellular Na⁺ and Ca²⁺ concentration.



like growth factor (IGF-I) or exercise (33, 35, 40, 49). On the other hand, class IB PI3K, which is linked to G protein-coupled receptors and is also capable of activating Akt, has been implicated in cardiac hypertrophy induced by pathological stress, such as pressure overload (39, 40, 42). Since ouabain

clearly activates the former, but not the latter, we have focused attention on ouabain effects through class IA PI3K.

How is class IA PI3K recruited to the plasma membrane, where ouabain interacts with Na⁺-K⁺-ATPase, to be activated? Since the most common process of activation of this

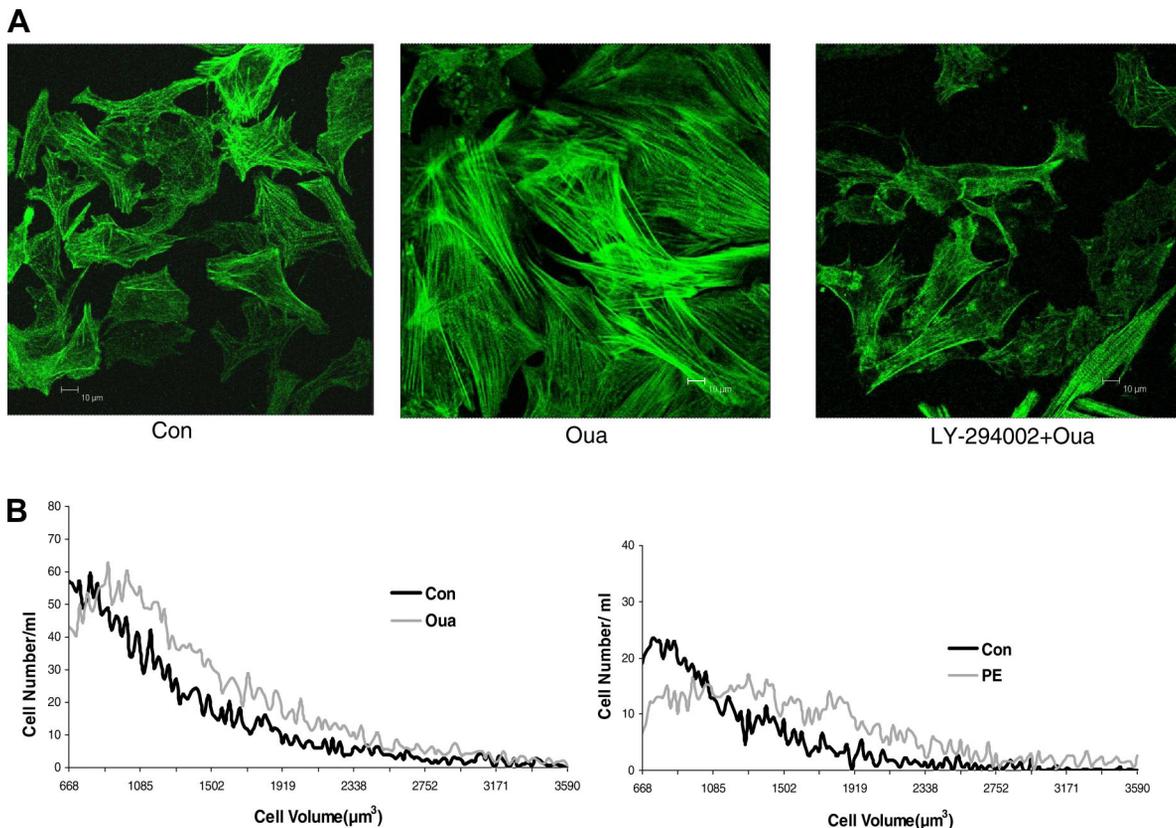


Fig. 8. Ouabain-induced hypertrophy of neonatal cardiac myocytes. *A*: cells were exposed to 100 μM ouabain without or with 2 μM LY-294002 for 48 h, stained with phalloidin, and visualized by confocal microscopy. *B*: cells were exposed to 100 μM ouabain or 100 μM phenylephrine (PE), a well-established hypertrophic stimulus in neonatal cardiac myocytes (19, 24, 43), as positive control, and cell number and volume were measured.

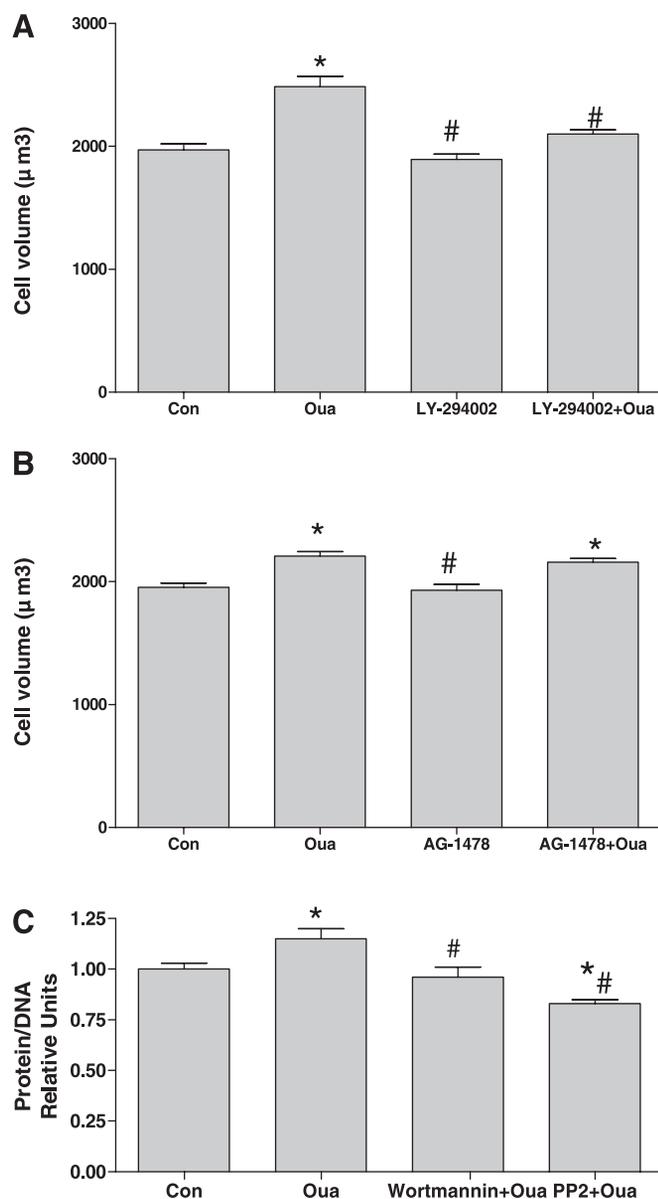


Fig. 9. Effects of various inhibitors on ouabain-induced hypertrophy of neonatal cardiac myocytes. Inhibitor concentrations were the same as those in Fig. 5. *A* and *B*: cell volume measured as described in Fig. 8*B* legend ($n = 6$). *C*: protein-to-DNA ratio ($n = 6$). * $P < 0.05$ vs. control. # $P < 0.05$ vs. Oua.

cytosolic PI3K involves its recruitment to the tyrosine-phosphorylated growth factor receptors of the plasma membrane (8, 40, 55) and since ouabain-induced phosphorylation of EGFR is established (17, 56), we explored the role of EGFR and found that it did not seem to be involved in the ouabain-induced activation of the PI3K-Akt pathway (Fig. 6). It is possible that ouabain may be transactivating other membrane receptor tyrosine kinases of cardiac myocytes, and this needs to be tested rigorously. However, in preliminary experiments on neonatal cardiac myocytes (not shown), we have been unable to establish the ouabain-induced tyrosine phosphorylation of the insulin receptor or the IGF-I receptor.

The most likely template for ouabain-induced recruitment of PI3K to the membrane is $\text{Na}^+\text{-K}^+\text{-ATPase}$. In their studies on dopamine-induced internalization of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in renal

epithelial cells, Yudowski et al. (58) were the first to suggest the interaction of the p85 regulatory subunit of class IA PI3K with the α -subunit of $\text{Na}^+\text{-K}^+\text{-ATPase}$ on the basis of immunoprecipitation studies and to provide evidence in support of such an interaction between the SH3 domain of p85 and a specific proline-rich domain of the α -subunit of $\text{Na}^+\text{-K}^+\text{-ATPase}$. Similar immunoprecipitation experiments have also been reported in subsequent studies on the roles of PI3K and $\text{Na}^+\text{-K}^+\text{-ATPase}$ in the regulation of epithelial cell motility (6), and our data of Fig. 4 are also consistent with the existence of this protein-protein interaction in cardiac myocytes. Since coimmunoprecipitation is never sufficient to establish direct interactions between two proteins, however, further studies on p85-ATPase interactions in the context of ouabain-induced signaling are needed. In this regard, it is appropriate to note that the "helical domains" of the p110 catalytic subunits of class I PI3Ks have also been implicated in protein-protein interactions, including those relevant to cardiac hypertrophy (38, 55).

Interrelations of the different ouabain-activated pathways. Ouabain-induced transactivation of EGFR requires Src (17, 18). If EGFR is not involved in the activation of the PI3K-Akt pathway by ouabain (Fig. 6), why is Src required for this activation? Perhaps ouabain-induced deinhibition of Src that is bound to $\text{Na}^+\text{-K}^+\text{-ATPase}$ is indeed the initial signaling event in all ouabain-induced signaling (53), and perhaps this deinhibited Src, still bound to $\text{Na}^+\text{-K}^+\text{-ATPase}$ (53), is necessary and sufficient for the recruitment/activation of PI3K. There is ample evidence for the direct activation of PI3K by Src (10, 11, 44). On the other hand, it is also possible that all or part of the Src effect on the ouabain-activated PI3K-Akt pathway may be downstream of PI3K. Src is known to phosphorylate Akt directly and to amplify the activation of Akt by PI3K and phosphoinositide-dependent kinase type 1 (20). Clearly, the mechanism of Src involvement in ouabain activation of the PI3K-Akt pathway also needs further study.

Our previous studies on ouabain-induced signaling in cardiac myocytes (31, 56) implied that the proximal events in these pathways were limited to $\text{Na}^+\text{-K}^+\text{-ATPase}$ interactions with Src-EGFR and the events that immediately emanate from EGFR. Evidently, this was an oversimplification, and it is necessary to include PI3K in the proximal ouabain-induced events as depicted in Fig. 7 in alternative ways that need to be tested.

It is important to emphasize that the apparent irrelevance of the EGFR-Ras-Raf-MEK-ERK cascade to ouabain-induced hypertrophy, as suggested by the data of Figs. 5 and 9, does not exclude the significant contribution of this ouabain-activated pathway and its branches to the overall signaling effects of the drug on the cardiac myocyte, or even to the PI3K-Akt-dependent hypertrophy. Ras is known to be involved in the activation of PI3K (8, 21, 52); hence, the ouabain-activated Ras, through EGFR, has the potential of direct interaction with PI3Ks (Fig. 7). There is ample evidence that ouabain effects on the EGFR-Ras-ERK cascade are also involved in increased mitochondrial reactive oxygen species generation (56, 57), the activations of several transcription factors (56), and transcriptional regulation of a number of genes (56), including those of the β -subunit of $\text{Na}^+\text{-K}^+\text{-ATPase}$ (26), which is critical to the compartmentalization of pumping and signaling functions of $\text{Na}^+\text{-K}^+\text{-ATPase}$ to cardiac myocyte caveolae (30). On the whole,

although the present findings reveal the essential role of the PI3K-Akt pathway in ouabain-induced hypertrophy, they also point to the previously unrecognized complexities of the ouabain-activated signaling pathways in cardiac myocytes.

Other implications. There is emerging evidence to suggest that activation of PI3K-Akt through class IA PI3K not only induces physiological hypertrophy, but it may also antagonize the potential detrimental effects of class IB PI3K signaling on cardiac function (12, 34). Although our present findings do not establish that ouabain-induced hypertrophy is identical to physiological hypertrophy, they are sufficient to justify the suggestion of such a working hypothesis, allowing exploration of the possibility that digitalis drugs may indeed ameliorate the consequences of the signaling pathways that are evoked by pressure overload. Although the clinical use of digitalis has declined in recent years, partly due to incomplete analysis of DIG trials (2, 16), recent studies clearly advocate the reversal of this inappropriate underuse of the drug (3, 4, 16). Delineating the newly appreciated signaling effects of digitalis in the heart, therefore, may have therapeutic implications.

GRANTS

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