

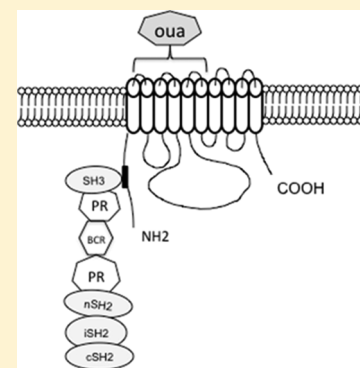
# Cell Signaling Associated with Na<sup>+</sup>/K<sup>+</sup>-ATPase: Activation of Phosphatidylinositide 3-Kinase IA/Akt by Ouabain Is Independent of Src

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**ABSTRACT:** Exposure of intact cells to selective inhibitors of Na<sup>+</sup>/K<sup>+</sup>-ATPase such as ouabain activates several growth-related cell signaling pathways. It has been suggested that the initial event of these pathways is the binding of ouabain to a preexisting complex of Src with Na<sup>+</sup>/K<sup>+</sup>-ATPase of the plasma membrane. The aim of this work was to evaluate the role of Src in the ouabain-induced activation of phosphatidylinositide 3-kinase IA (PI3K1A) and its downstream consequences. When fibroblasts devoid of Src (SYF cells) and controls (Src<sup>++</sup> cells) were exposed to ouabain, PI3K1A, Akt, and proliferative growth were similarly stimulated in both cell lines. Ouabain-induced activation of Akt was not prevented by the Src inhibitor PP2. In contrast, ERK1/2 were not activated by ouabain in SYF cells but were stimulated in Src<sup>++</sup> cells; this was prevented by PP2. In isolated adult mouse cardiac myocytes, where ouabain induces hypertrophic growth, PP2 also did not prevent ouabain-induced activation of Akt and the resulting hypertrophy. Ouabain-induced increases in the levels of co-immunoprecipitation of the  $\alpha$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase with the p85 subunit of PI3K1A were noted in SYF cells, Src<sup>++</sup> cells, and adult cardiac myocytes. In conjunction with previous findings, the results presented here indicate that (a) if there is a preformed complex of Src and Na<sup>+</sup>/K<sup>+</sup>-ATPase, it is irrelevant to ouabain-induced activation of the PI3K1A/Akt pathway through Na<sup>+</sup>/K<sup>+</sup>-ATPase and (b) a more likely, but not established, mechanism of linkage of Na<sup>+</sup>/K<sup>+</sup>-ATPase to PI3K1A is the ouabain-induced interaction of a proline-rich domain of the  $\alpha$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase with the SH3 domain of the p85 subunit of PI3K1A.



Na<sup>+</sup>/K<sup>+</sup>-ATPase is the energy-transducing enzyme of the plasma membrane that catalyzes the coupled active transport of Na<sup>+</sup> and K<sup>+</sup> in most higher eukaryotic cells.<sup>1,2</sup> Two subunits of the enzyme ( $\alpha$  and  $\beta$ ) are essential for this pumping function,<sup>2</sup> but the  $\alpha$ -subunit contains the ATP binding site and the ion transport pathways.<sup>3</sup> Many preparations of the enzyme from different cell types also contain a third subunit (FXYP protein) that regulates function.<sup>2</sup>

In addition to its essential ion pumping function, Na<sup>+</sup>/K<sup>+</sup>-ATPase may also act as a signal transducer. When intact cells are exposed to digitalis drugs that are known to be highly specific inhibitors of Na<sup>+</sup>/K<sup>+</sup>-ATPase (e.g., ouabain, digoxin, and digitoxin), a number of intracellular signaling pathways are activated, leading to highly cell specific downstream consequences.<sup>4,5</sup> To date, two ouabain-activated pathways that are growth-related have been identified in a variety of cell types: the EGFR/Src–Ras–ERK pathway and the PI3K1A–PDK–Akt pathway.<sup>4,6</sup> In cells that are capable of proliferative growth, ouabain-induced signaling causes either stimulation or inhibition of growth depending on the cell type,<sup>7,8</sup> with unclear downstream mechanisms for either growth stimulation or inhibition.<sup>7,9</sup> In the terminally differentiated cardiac myocytes where nontoxic concentrations of ouabain cause hypertrophic growth, the two pathways are activated in parallel, but only the

PI3K1A–PDK–Akt pathway seems to be relevant to ouabain-induced hypertrophy.<sup>6,10</sup>

Ouabain activation of the EGFR/Src–Ras–ERK signaling pathway was the first to be discovered;<sup>11,12</sup> hence, a significant amount of work on how it may be linked to Na<sup>+</sup>/K<sup>+</sup>-ATPase has been conducted. On the basis of the original observations of Tian et al.,<sup>13</sup> a large body of subsequent research has advanced the hypothesis that the initial event of this drug-induced signaling is due to a normal preexisting pool of inactive Src that is bound to intracellular domains of the  $\alpha$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase, and that binding of ouabain to the extracellular domains of the  $\alpha$ -subunit leads to the disinhibition of this Src, allowing the stimulation of the EGFR/Src–Ras–ERK pathway and its downstream growth consequences.

There is a paucity of experimental data about the mechanism through which the ouabain-inhibited Na<sup>+</sup>/K<sup>+</sup>-ATPase may lead to the activation of PI3K1A. Nevertheless, because of the repeated advocacy of the hypothesis that a preformed complex of Src and Na<sup>+</sup>/K<sup>+</sup>-ATPase is the receptor for all ouabain-induced signaling,<sup>14–20</sup> it has been tacitly assumed that this postulated Src–Na<sup>+</sup>/K<sup>+</sup>-ATPase complex also initiates the

Received: August 27, 2013

Revised: November 13, 2013

Published: November 15, 2013

ouabain activation of cell signaling through PI3K1A.<sup>8,21</sup> The primary aims of this work were the testing of this assumption and the clarification of the mechanisms of drug-induced cell signaling through the ubiquitous Na<sup>+</sup>/K<sup>+</sup>-ATPase.

## MATERIALS AND METHODS

**Cell Lines.** SYF cells, deficient for tyrosine kinases Src, Yes, and Fyn, and Src<sup>++</sup> cells, a control expressing endogenous wild-type Src but lacking expression of Yes and Fyn, were mouse fibroblasts obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). When cultures reached approximately 80–90% confluence, cells were serum-starved overnight before being used for the signaling experiments.

**Adult Mouse Cardiomyocyte Culture.** Isolation and culture of adult cardiomyocytes from cardiac specific Na<sup>+</sup>/Ca<sup>2+</sup> exchange knockout mice were performed following the procedures previously described.<sup>10,21</sup> For the cell signaling experiment, myocytes were cultured in MEM containing 2,3-butanedione monoxime (BDM) and 1 mg/mL BSA overnight and then transferred to the same medium without BDM before the indicated treatments.

**SDS-PAGE and Western Blot Analysis.** This was performed as previously described.<sup>6</sup> Briefly, cells were washed with cold phosphate-buffered saline (PBS) and lysed in modified radioimmune precipitation assay (RIPA) buffer containing 1 mM NaF, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 nM okadaic acid. Cell lysates were rotated for 30 min at 4 °C and centrifuged at 14000 rpm for 15 min. Samples were subjected to 8 or 10% SDS-PAGE, transferred to PVDF membrane, and probed with appropriate antibodies by standard procedures. The immunoreactive bands were developed and detected using enhanced chemiluminescence. For quantitative comparisons, images were scanned with a densitometer. Different dilutions of samples were subjected to SDS-PAGE, and multiple exposures of the films were used to ensure that quantifications were made within the linear range of the assays. The primary antibodies and their sources were as follows: anti-cSrc [sc-18 (Santa Cruz Biotechnology, Santa Cruz, CA)], anti-PI3-kinase p110α [611398 (BD Biosciences)], anti-PI3-kinase p85α [06-497 (EMD Millipore Corp.)], anti-phospho-Akt [9271 (Cell Signaling Technology, Inc.)], anti-Akt [9272 (Cell Signaling Technology, Inc.)], anti-phospho-ERK [9106 (Cell Signaling Technology, Inc.)], anti-ERK [sc-94 (Santa Cruz Biotechnology)], and anti-GAPDH [sc-20357 (Santa Cruz Biotechnology)]. The horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

**PI3K1A Kinase Activity Assay.** Src<sup>++</sup> cells, SYF cells, and mouse cardiomyocytes were cultured in 100 mm dishes. Each sample was collected from three dishes, and the assay was conducted as described previously.<sup>6</sup> Briefly, cells were lysed with ice-cold buffer containing 140 mM NaCl, 10 mM HEPES, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10% glycerol, 1% Nonidet P-40, 10 μg/mL aprotinin, 50 μM leupeptin, and 2 mM PMSF (pH 8.1) and solubilized by continuous rotation for 1 h at 4 °C. After centrifugation (16000g for 15 min), the supernatant was collected, and equal amounts of protein (0.5–2 mg) from control and ouabain-treated cells were incubated with an anti-

PI3K p85α antibody. After overnight incubation, protein A-agarose was added, and the immune complex was washed four times with buffer [100 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 20 mM HEPES (pH 7.5)] and resuspended in 40 μL of buffer [180 mM NaCl and 20 mM HEPES (pH 7.5)]. PI3K activity in the immunoprecipitates was assayed directly on the beads by a standard procedure with phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) and [ $\gamma$ -<sup>32</sup>P]ATP used as substrates. The reactions were performed at room temperature and stopped after 10 min by addition of 80 μL of 1 M HCl. The lipids were extracted with 160 μL of a chloroform/methanol mixture (1:1), spotted on a thin-layer chromatography plate, and separated with a chloroform/acetone/methanol/glacial acetic acid/H<sub>2</sub>O mixture (40:15:13:12:8). The radioactivity of the phosphorylated lipid product PIP<sub>3</sub> was quantified by a PhosphorImager (Molecular Dynamics).

**Phosphatidylinositol 3,4,5-Triphosphate (PIP<sub>3</sub>) Immunostaining.** SYF and Src<sup>++</sup> cells cultured on coverslips were fixed with 2% paraformaldehyde in PBS and permeabilized with 0.5% saponin. After being blocked with 10% goat serum in PBS for 15 min in room temperature, cells were incubated with mouse anti-PIP<sub>3</sub> IgM [Z-P345 (Echelon Biosciences)] overnight at 4 °C followed by incubation with secondary antibody Alexa 488 anti-mouse for 1 h at 37 °C. Cell nuclei were then stained with 0.5 μg/μL propidium iodide [P21493 (Molecular Probes, Invitrogen Detection Technologies)] for 5 min. The fluorescence-labeled cells on microscope slides were mounted with ProLong Gold Antifade reagent [P36930 (Life Technologies Corp., Invitrogen)] and sealed with nail polish. Cell images were acquired with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany), and the average intensity of PIP<sub>3</sub> was normalized by the number of nuclei.

**Co-Immunoprecipitation.** SYF and Src<sup>++</sup> cells were lysed in RIPA buffer. The total amount of 1.2 mg of protein as incubated with anti-PI3-kinase p85α antibody [06-195 (EMD Millipore Corp.)] overnight at 4 °C. Protein A agarose beads were added for 3 h at 4 °C. The bead-bound immunocomplexes were eluted in the sample loading buffer at 55 °C for 20 min. The supernatants were collected and then were subjected to SDS-PAGE for Western blot analysis. The following primary antibodies were used: anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase α1 antibody [α6F (Department of Biological Science, The University of Iowa, Iowa City, IA)] and anti-PI3-kinase p85α [sc-1637 (Santa Cruz Biotechnology)].

**Fibroblast Proliferation Assay.** SYF and Src<sup>++</sup> cells were seeded in six-well plates at a density of 25000 cells per well in DMEM containing 10% FBS for 24 h. Then the cells were incubated with 1% FBS overnight and then with and without ouabain for an additional 12 h. After cells had been detached with 0.25% trypsin-EDTA [25200-056 (Life Technologies Corp.)], cell numbers were counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Inc.).

**Rate of Protein Synthesis as a Measure of Hypertrophy in Cardiomyocytes.** This was conducted using a [<sup>3</sup>H]leucine incorporation assay as described previously.<sup>10</sup>

**Statistical Analysis of Data.** Data are expressed as mean ± the standard error of the mean. All analyses were performed on SPSS version 17.0 (SPSS Inc., Chicago, IL). A *P* value of <0.05 was considered statistically significant.

All research on cardiac specific Na<sup>+</sup>/Ca<sup>2+</sup> exchange knockout mice was conducted according to procedures and guidelines approved by the Institutional Animal Care and Use Committee.

RESULTS

Ouabain-Induced Activation of PI3K1A in SYF Cells.

This mouse embryonic fibroblast cell line lacking expression of Src, Yes, and Fyn has been used in previous studies to explore the role of Src in ouabain-induced stimulation of the EGFR/Src-Ras-ERK pathway.<sup>13,22</sup> In the following experiments, therefore, we used the SYF and control Src<sup>++</sup> cells to investigate the relation of Src to ouabain-induced activation of PI3K1A.

We compared the basal levels of several signal pathway proteins that are relevant to this study in SYF and Src<sup>++</sup> cells using Western blots. As expected, Src was not detected in SYF cells, but the basal levels of Akt, the regulatory and catalytic subunits of PI3K1A, and ERK1/2 were not significantly different in the SYF and control cells (Figure 1). We then

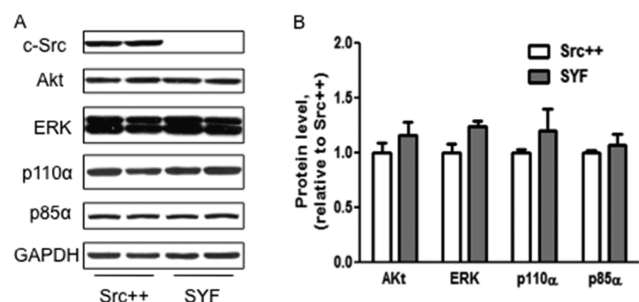


Figure 1. Basal levels of Src, Akt, ERKs, and p85 and p110α subunits of PI3K1A in SYF and Src<sup>++</sup> cells. (A) Representative blots of the proteins immunoassayed as indicated in Materials and Methods. (B) Quantitative comparisons of the blot intensities normalized by GAPDH (n = 3–6).

exposed intact SYF and Src<sup>++</sup> cells to ouabain for 5 min, immunisolated PI3K1A from the lysates, and assayed its activity by a standard procedure, measuring the product (PIP3) on TLC plates (Figure 2). The results indicated that ouabain treatment of the cells activated PI3K1A in both SYF and control cells. To see if this finding could be confirmed by a different assay, the intact cells that were similarly exposed to ouabain were fixed and stained with an anti-PIP3 antibody (Figure 3). The results confirmed that the level of PIP3 increased in both SYF and Src<sup>++</sup> cells upon exposure to ouabain (Figure 3A,B). Interestingly, the basal level of PIP3 prior to ouabain exposure was lower in SYF cells than in Src<sup>++</sup> cells (Figure 3C).

Consequences of Ouabain-Induced Activation of PI3K1A in SYF Cells.

The findings described above prompted us to determine if Src deletion affected the ouabain activation of the entire PI3K1A-PDK-Akt pathway and its growth consequences in SYF cells. The results of the experiments of Figure 4 showed that ouabain activated Akt in both SYF and Src<sup>++</sup> cells and that Src inhibitor PP2 did not prevent the ouabain activation of Akt in Src<sup>++</sup> cells. In the experiments depicted in Figure 5, we compared the effects of ouabain on the proliferative growth of the SYF and Src<sup>++</sup> cells. Because of the known existence of signal amplification mechanisms for ouabain effects on cell proliferation,<sup>23</sup> and due to biphasic effects of ouabain (stimulation followed by inhibition) in cell proliferation studies,<sup>24</sup> in conducting the experiments depicted in Figure 5, we first determined by preliminary experiments (data not shown) the optimal ouabain concentration (100 nM) for stimulation and then compared its effects on the two cell lines. The results showed significant stimulation by ouabain in

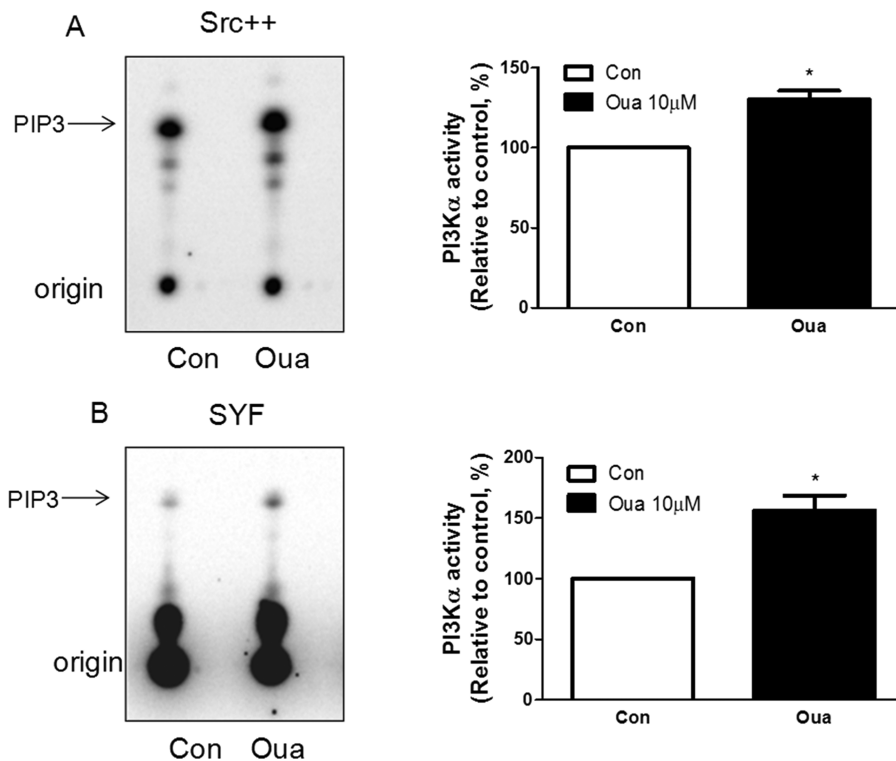
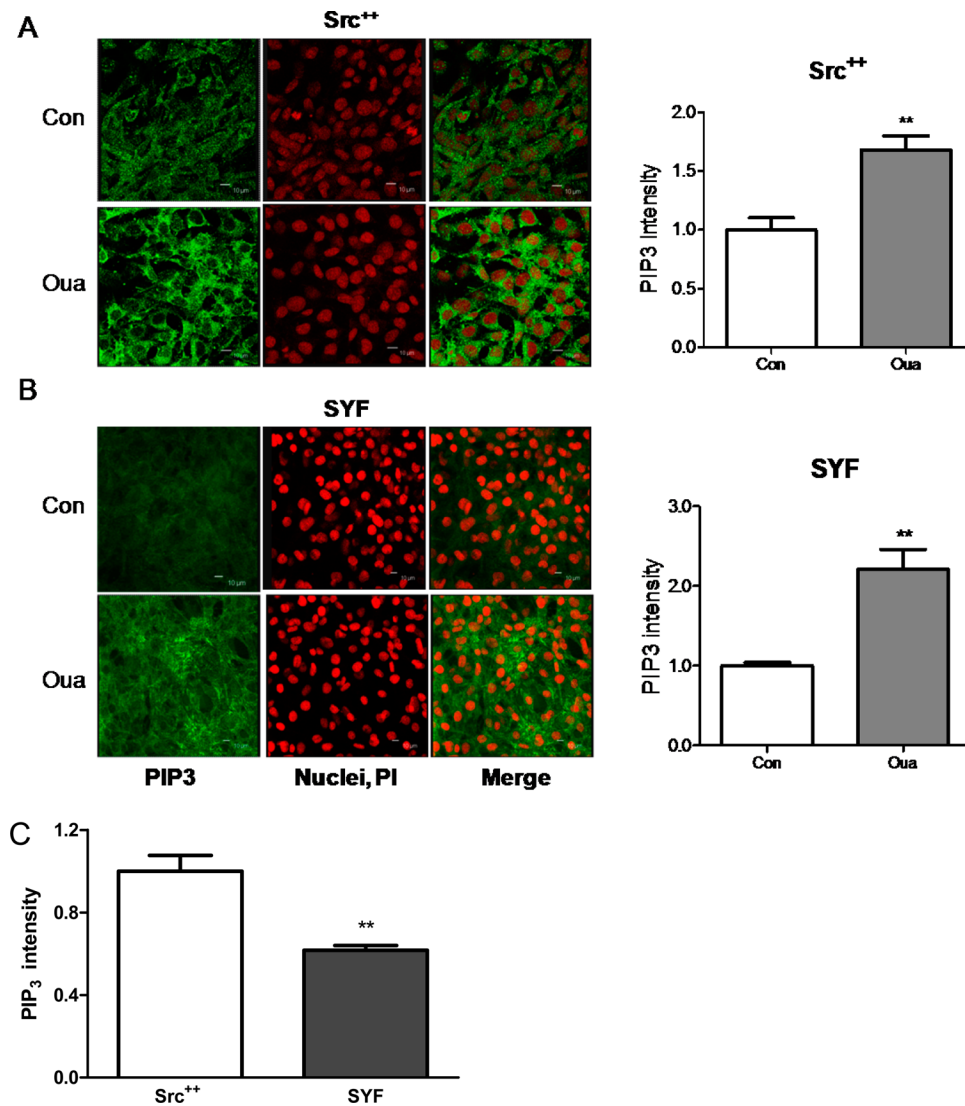


Figure 2. Effect of ouabain on PI3K1A activity in Src<sup>++</sup> and SYF cells. Cells were treated with 10 μM ouabain for 5 min, and then lysates were used for the kinase assay as indicated in Materials and Methods: (A) Src<sup>++</sup> cell and (B) SYF cell (\*P < 0.05 vs control; n = 3). Equal amounts of lysate proteins were subjected to the assay from the lysates of the control cells and those treated with ouabain. The amount of lysates used for the assay varied, however, in different experiments depicted in panels A and B.



**Figure 3.** Effect of ouabain on PIP3 levels in Src<sup>++</sup> and SYF cells. Cells were treated with 10 μM ouabain for 5 min and then fixed for PIP3 staining: (A) Src<sup>++</sup> cell and (B) SYF cell. (C) Basal level of PIP3. The average intensities of PIP3 (green) were normalized by the numbers of cell nuclei (red) (\*\**P* < 0.01 vs control; *n* = 5).

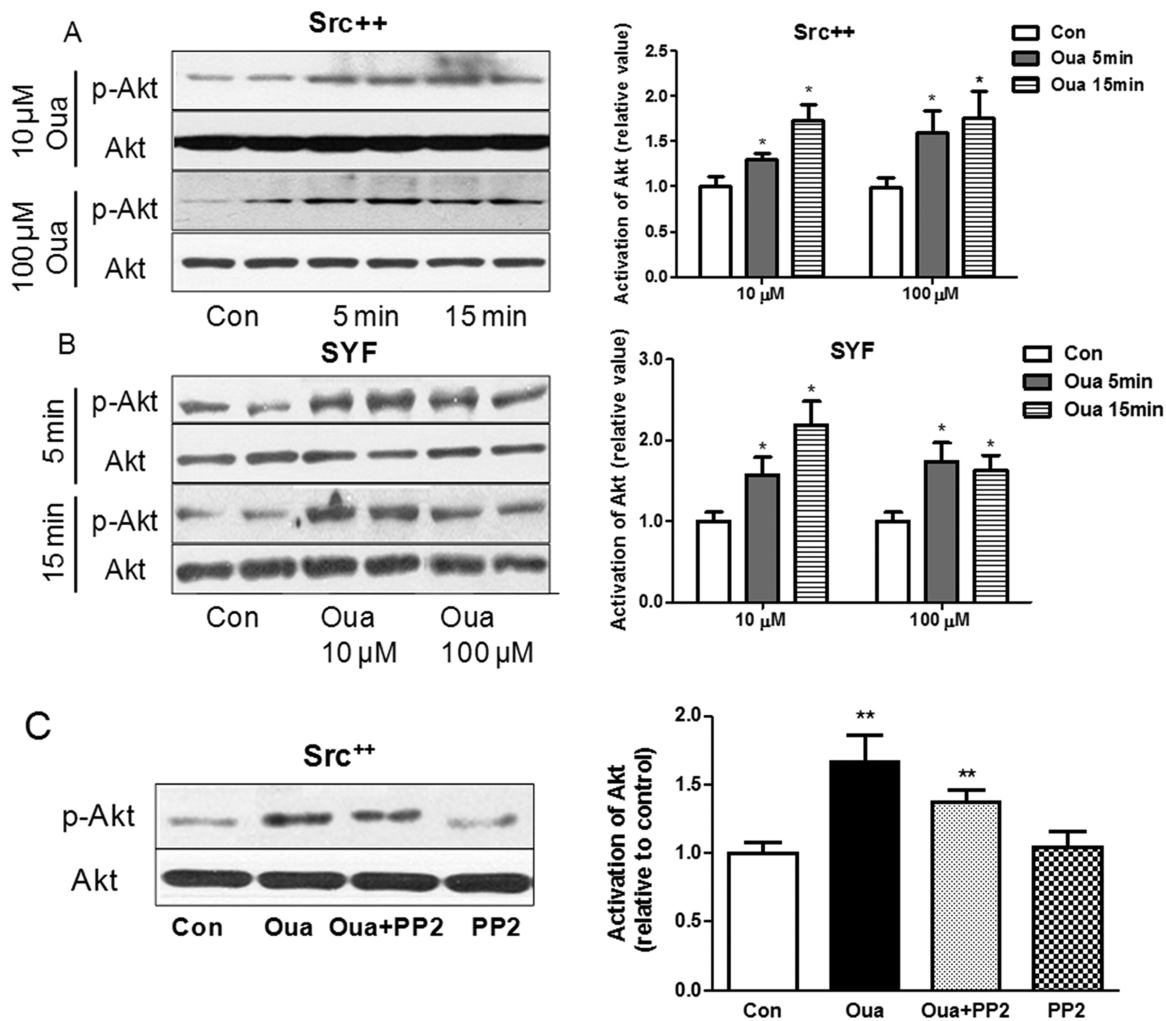
both cell lines. The combined data of Figures 4 and 5 indicated that Src was not necessary for ouabain activation of the PI3K1A–PDK–Akt pathway and the resulting increase in the level of proliferation of these fibroblasts.

**Effects of Ouabain on ERK1/2 in SYF and Src<sup>++</sup> Cells.** Previous studies that have been interpreted to support the necessity of Src for ouabain-induced activations of ERKs were conducted with SYF and SYF+Src cells that strongly over-express Src.<sup>13,22</sup> To see if similar results could be obtained with Src<sup>++</sup> cells that contained normal Src levels, we performed the experiments depicted in Figure 6. Ouabain did not activate ERKs in SYF cells but caused activation in Src<sup>++</sup> cells. This activation was prevented by the Src inhibitor PP2 (Figure 6).

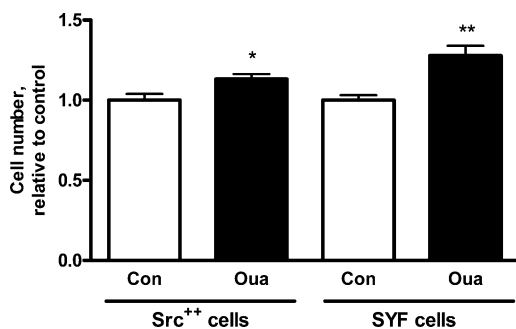
**Effects of Src Inhibition on Ouabain-Induced Activation of Akt and Increase in the Level of Protein Synthesis in Adult Mouse Cardiac Myocytes.** To explore the role of Src in ouabain activation of the PI3K1A–PDK–Akt pathway and the resulting hypertrophic growth, we used terminally differentiated isolated adult mouse cardiac myocytes. For these experiments, myocytes isolated from Na/Ca exchanger 1 (NCX1) knockout mouse hearts, in which ouabain

induces hypertrophy but no increase in contractility,<sup>10</sup> were used. The experiments depicted in Figure 7 showed that pretreatment with Src inhibitor PP2 did not prevent ouabain-induced activation of Akt in myocytes of NCX1 knockout mice. The results of the experiments depicted in Figure 8 indicated that PP2 was also ineffective in blocking the ouabain-induced increase in the rate of protein synthesis in isolated myocytes. The findings depicted in Figures 7 and 8 strengthened the conclusions of the experiments with SYF cells, suggesting the redundancy of Src to ouabain-induced activation of the PI3K1A–PDK–Akt pathway and its growth consequences.

**Effects of Ouabain on the Interaction of the α-Subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase with the p85 Subunit of PI3K1A in SYF Cells, Src<sup>++</sup> Cells, and Adult Mouse Cardiac Myocytes.** Ouabain-activated interaction between Na<sup>+</sup>/K<sup>+</sup>-ATPase and PI3K 1A was detected previously in neonatal rat cardiac myocytes,<sup>6</sup> by co-immunoprecipitation experiments. We conducted similar studies on the mouse cells used here. Ouabain similarly increased the level of p85 immunoprecipitation with the α-subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase in SYF cells, Src<sup>++</sup> cells, and adult myocytes (Figure.9),



**Figure 4.** Effect of ouabain on Akt activation in Src<sup>++</sup> (A) and SYF (B) cells. Cells were exposed to ouabain for 5 and 15 min. In separate experiments (C), Src<sup>++</sup> cells were incubated with or without PP2 (2 μM) for 30 min prior to ouabain (10 μM) treatment. Akt activations were expressed by ratio of p-Akt to total Akt (\**P* < 0.05 and \*\**P* < 0.01 vs control; *n* = 6–11).



**Figure 5.** Effect of ouabain on cell proliferation in Src<sup>++</sup> and SYF cells. Cells were incubated with 100 nM ouabain in the presence of 1% FBS for 12 h. Cell numbers were counted as described in Materials and Methods (\**P* < 0.05 and \*\**P* < 0.01 vs control; *n* = 9 for Src<sup>++</sup>; *n* = 6 for SYF).

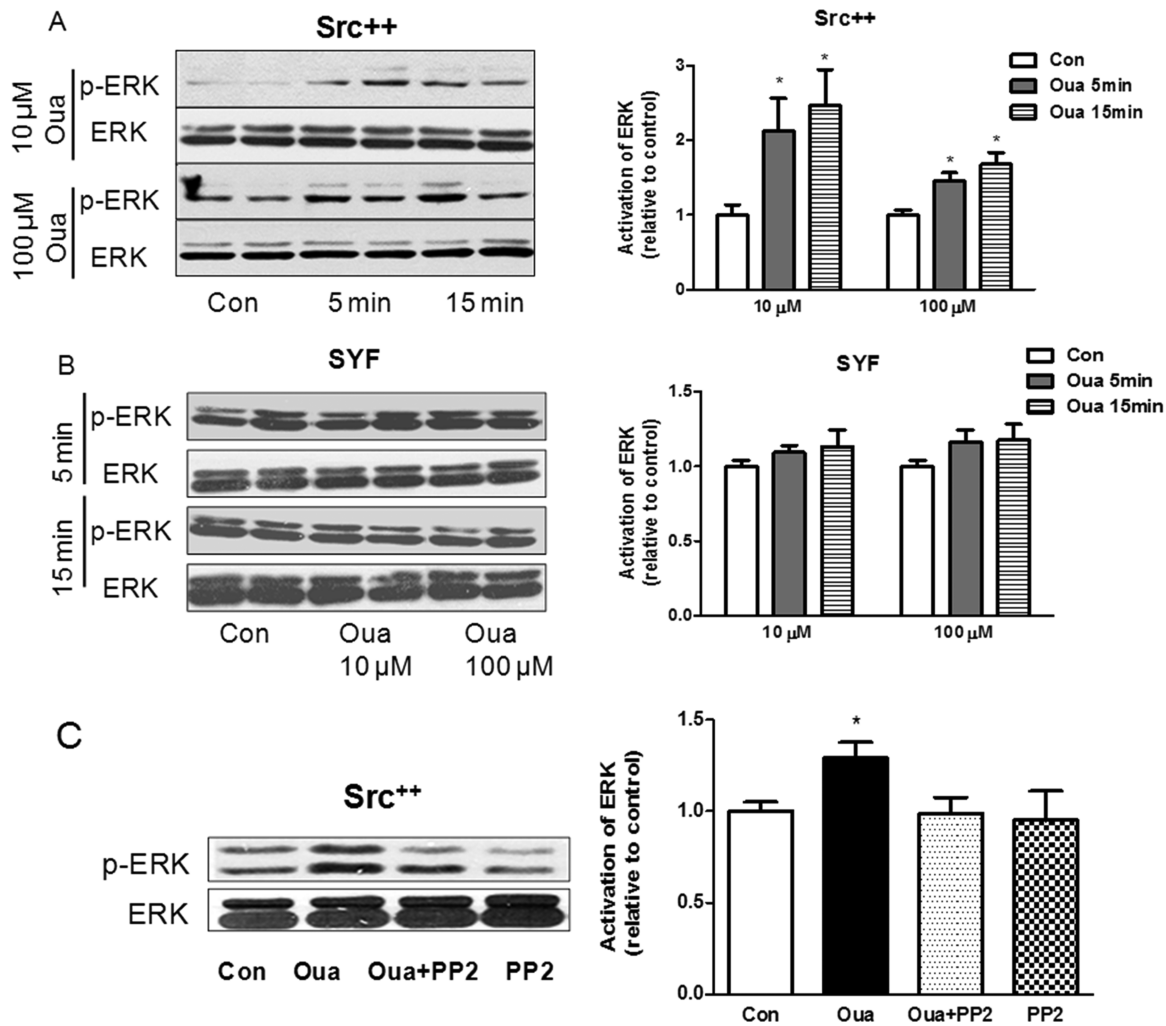
indicating the irrelevance of Src to this protein–protein interaction.

**DISCUSSION**

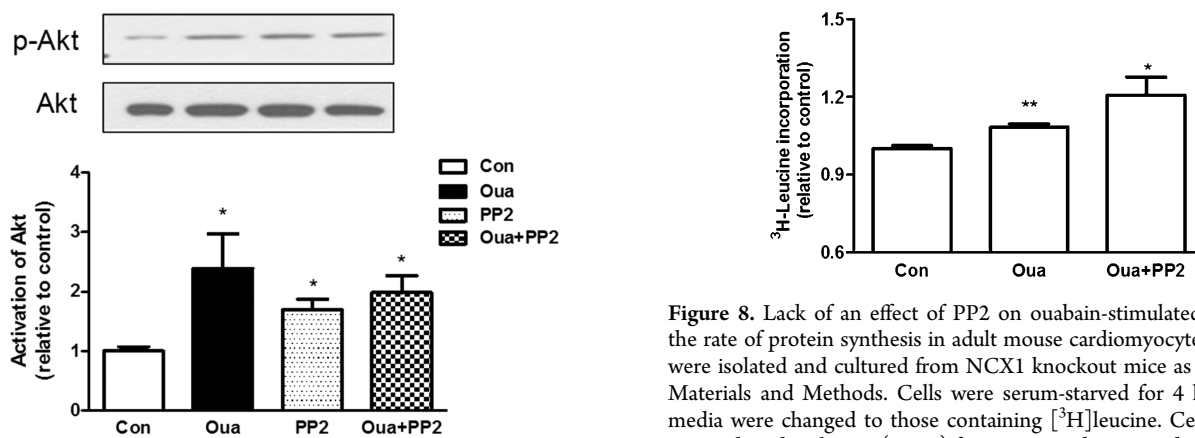
Activation of PI3K1A by ouabain was first reported in renal epithelial cells in relation to control of apoptosis,<sup>25</sup> then in the same cells in relation to internalization of Na<sup>+</sup>/K<sup>+</sup>-ATPase at

the clathrin-coated pits,<sup>26</sup> and subsequently in cardiac myocytes,<sup>6,10</sup> where ouabain induces hypertrophic growth that seems to be similar to beneficial physiological hypertrophy. It is important, therefore, to attempt to clarify the mechanism by which the ouabain-inhibited Na<sup>+</sup>/K<sup>+</sup>-ATPase is linked to activation of PI3K1A.

PI3K1A is an obligatory dimer of p85 and p110, and its activation reflects the reversal of the inhibitory effect of p85 on the catalytic activity of p110 without the dissociation of the dimer.<sup>27,28</sup> The most common way of disinhibiting the kinase is through binding of its p85-SH2 domains to the tyrosine-phosphorylated motifs of the receptor tyrosine kinases.<sup>28,29</sup> There are, however, several other established ways in which this disinhibition may occur.<sup>27–29</sup> One such alternative mechanism is the direct binding of the Src-SH3 domain to the proline-rich domains of the p85 subunit of PI3K1A. This has been demonstrated to be the mechanism of activation of PI3K1A by some extracellular signals.<sup>28</sup> We were prompted to test if Src is similarly involved in ouabain-induced activation of PI3K1A for two reasons (1) because the complex of Src with Na<sup>+</sup>/K<sup>+</sup>-ATPase has been proposed to be the initial target of all signaling initiated by ouabain and related digitalis drugs<sup>13,20</sup> and (2) because recent evidence has led to questions about whether there is direct contact between Src and Na<sup>+</sup>/K<sup>+</sup>-ATPase.<sup>30,31</sup>



**Figure 6.** Effect of ouabain on ERK activation in Src<sup>++</sup> (A) and SYF cells (B). Cells were exposed to ouabain for 5 and 15 min. In separate experiments (C), Src<sup>++</sup> cells were incubated with or without PP2 (2  $\mu$ M) for 30 min prior to ouabain (10  $\mu$ M) treatment. ERK activation was assayed by the ratio of phosphorylated ERK to total ERK (\**P* < 0.05 vs control; *n* = 6–11).

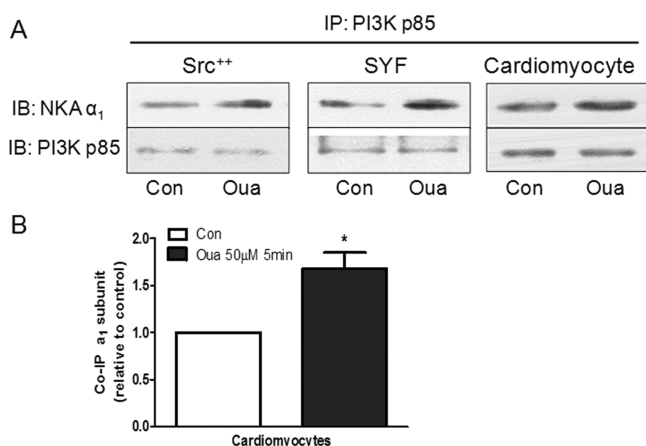


**Figure 7.** Ouabain-induced activation of Akt and lack of effect of PP2 on ouabain in adult mouse cardiomyocytes. Myocytes were isolated and cultured from NCX1 knockout mice as described in Materials and Methods. Cells were preincubated with PP2 (2  $\mu$ M) for 30 min. PP2-treated and untreated cells were then incubated with ouabain (50  $\mu$ M) for 5 min (\**P* < 0.05 vs control; *n* = 5).

**Figure 8.** Lack of an effect of PP2 on ouabain-stimulated increase in the rate of protein synthesis in adult mouse cardiomyocytes. Myocytes were isolated and cultured from NCX1 knockout mice as described in Materials and Methods. Cells were serum-starved for 4 h before the media were changed to those containing [<sup>3</sup>H]leucine. Cells that were preincubated with PP2 (2  $\mu$ M) for 30 min and untreated controls were incubated with ouabain (10  $\mu$ M) for 12 h (\**P* < 0.05 and \*\**P* < 0.01 vs control; *n* = 4).

The mouse embryonic fibroblast cells lacking Src, Yes, and Fyn (SYF cells) have been used extensively to determine the

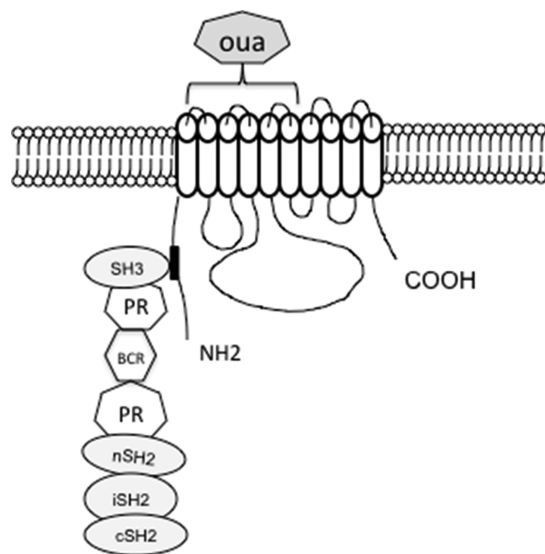
possible involvement of Src in the activation of PI3K1A by various extracellular stimuli.<sup>32–34</sup> SYF cells have also been used to assess the role of Src in ouabain-induced activation of ERKs through the EGFR/Src–Ras–ERK1/2 pathway.<sup>13,22</sup> In these prior studies, SYF+Src cells in which Src was overexpressed



**Figure 9.** Ouabain-induced co-immunoprecipitation of the  $\alpha 1$  subunit of Na/K-ATPase with the p85 subunit of PI3K1A in Src<sup>+</sup> cells, SYF cells, and cardiomyocytes. (A) Representative blots of two experiments with Src<sup>+</sup> and SYF cells and three experiments with myocytes. (B) Quantitative data from cardiomyocytes (\**P* < 0.05 vs control).

were used. In the studies presented here, using SYF cells and the appropriate control Src<sup>+</sup> cells, we have now confirmed the necessity of Src for ouabain-induced activation of the EGFR/Src–Ras–ERK1/2 pathway (Figure 6), but we have also clearly established that ouabain-induced activation of the PI3K1A–PDK–Akt pathway is independent of Src (Figures 1–4). Our findings also show that the Src-independent activation of PI3K1A and Akt by ouabain leads to stimulation of proliferative growth in fibroblasts (Figure 5) and hypertrophic growth in cardiac myocytes (Figure 8).

If Src is unnecessary for the ouabain-induced activation of PI3K1A, what is the mechanism of the linkage between Na<sup>+</sup>/K<sup>+</sup>-ATPase and PI3K1A? The co-immunoprecipitation experiments, both in fibroblasts and in cardiac myocytes (Figure 9), suggest that the more likely mechanism is the direct interaction of the p85-SH3 domain with a proline-rich domain of the  $\alpha$ -subunit of the ouabain-inhibited Na<sup>+</sup>/K<sup>+</sup>-ATPase (Figure 10). This hypothesis is supported by numerous studies showing that



**Figure 10.** Postulated domain–domain interactions of the  $\alpha$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase and the p85 subunit of PI3K1A.

PI3K1A may indeed be activated by interactions of p85-SH3 domains with proline-rich domains of several other proteins,<sup>27,28</sup> and by previous data suggesting that a specific proline-rich domain of the  $\alpha$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase (TPPPTTP<sup>87</sup>) may be involved in dopamine-induced internalization of Na<sup>+</sup>/K<sup>+</sup>-ATPase at the clathrin-coated pits.<sup>35</sup> However, the working hypothesis of Figure 10 is far from being established because of the known limitations of the supporting co-immunoprecipitation studies, i.e., the possibility that the detergents used in such studies may be inducing the noted interaction between the denatured  $\alpha$ -subunit and the native PI3K1A. Further testing of the hypothesis is needed.

Regardless of the main implication of these studies, the following aspects of our findings require further discussion.

In our studies on the effects of ouabain on SYF and Src<sup>+</sup> cells, the ouabain concentration (100 nM) used to detect the drug’s stimulation of proliferation in these fibroblasts (Figure 5) is significantly lower than the ouabain concentrations (10–100  $\mu$ M) used in the signaling experiments (Figures 2–4). Such seeming discrepancies between the ouabain sensitivities of the long-term proliferation experiments and the short-term signaling experiments have been noted previously<sup>23</sup> and are caused by signal amplification between receptor occupancy and the far downstream growth effects.<sup>23</sup> The suggested mechanisms of this amplification are yet to be explored.

With regard to the ouabain concentrations (10–100  $\mu$ M) used in the signaling experiments with these fibroblast cell lines, it is appropriate to ask if the use of such high drug concentrations is consistent with the early data and conclusions<sup>4,36</sup> that ouabain-induced signaling occurs in a manner independent of changes in intracellular ion concentrations. Fibroblasts contain only the  $\alpha 1$  isoform of Na<sup>+</sup>/K<sup>+</sup>-ATPase, and the *K*<sub>0.5</sub> value for the ouabain inhibition of this rodent isoform is  $\geq 100 \mu$ M.<sup>37</sup> Considering the long-established reserved capacity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase in a variety of cell types,<sup>36,38,39</sup> showing that  $\sim 50\%$  inhibition of the pump is needed before significant changes in normal Na<sup>+</sup>/K<sup>+</sup> gradients occur, it is likely that in our present short-term signaling experiments, there is also little or no ouabain-induced change in Na<sup>+</sup>/K<sup>+</sup> gradients.

Finally, it is of special interest that in experiments depicted in Figure 3 where cellular PIP3 contents were assayed by immunostaining, we noted (Figure 3C) that prior to ouabain exposure, the basal level of PIP3 was lower in SYF cells than in Src<sup>+</sup> cells. This suggests that in spite of the irrelevance of Src with respect to ouabain-induced activation of PI3K1A, Src family kinases may be involved in the regulation of the steady state levels of PIP3 by an unknown mechanism, perhaps through the control of the lipid phosphatases that degrade PIP3.

In conclusion, these studies clearly rule out the presumed role of Src in ouabain-induced activation of PI3K1A and point to the necessity of focusing on the alternative mechanisms by which digitalis drugs activate PI3K1A and regulate several important physiological processes.

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## Funding

This study was supported by National Heart, Lung, and Blood Institute Grant PO1 HL-36573.

## Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We thank Jenifer Zak for capable assistance in the preparation of the manuscript. We also thank Marjorie Gable and Shadi Zahedi for excellent technical support.

## ABBREVIATIONS

BDM, 2,3-butanedione monoxime; HRP, horseradish peroxidase; NCX1, Na/Ca exchanger 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-triphosphate; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay.

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