Different roles of the cardiac Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger in ouabain-induced inotropy, cell signaling, and hypertrophy

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Am J Physiol Heart Circ Physiol 304: H427–H435, 2013. First published November 30, 2012; doi:10.1152/ajpheart.00462.2012.—Previous studies have shown that digitalis drugs, acting as specific inhibitors of cardiac Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, not only cause positive inotropic effects, but also activate cell signaling pathways that lead to cardiac myocyte hypertrophy. A major aim of this work was to assess the role of Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger, NCX1, in the above two seemingly related drug effects. Using a mouse with ventricular-specific knockout (KO) of NCX1, ouabain-induced positive inotropy that was evident in wild-type (Wt) hearts was clearly reduced in KO hearts. Ouabain also increased Ca\textsuperscript{2+} transient amplitudes in Wt myocytes, but not in KO myocytes. Ouabain-induced activations of ERK 1/2 were noted in Wt myocytes, but not in KO myocytes; however, ouabain activated PI3K1A and Akt in both Wt and KO myocytes. Protein synthesis rate, as a measure of hypertrophy, was increased by ouabain in Wt and KO myocytes; these drug effects were prevented by a PI3K inhibitor but not by a MEK/ERK inhibitor. Hypertrophy caused by ET-1, but not that induced by ouabain, was accompanied by upregulation of BNP gene in Wt and KO myocytes. The findings indicate 1) the necessity of NCX1 for positive inotropic action of ouabain; 2) the irrelevance of NCX1 and ERK 1/2 activation to ouabain-induced hypertrophy; and 3) that hypertrophy caused by ouabain through the activation of PI3K1A/Akt pathway is likely to be beneficial to the heart.

PI3K; cardiac hypertrophy; positive inotropy; calcium

NA\textsuperscript{+}/K\textsuperscript{+}-ATPase is an oligomeric membrane protein, consisting of α, β, and FXYD subunits, that catalyzes the coupled active transport of Na\textsuperscript{+} and K\textsuperscript{+} across the plasma membranes of most mammalian cells, including cardiac myocytes (12, 35). This ion pumping function of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase is inhibited specifically by digitalis drugs and related cardiac glycosides such as digoxin, digitoxin, and ouabain (35, 36).

In the heart, the long-established positive inotropic effects of digitalis drugs, which were demonstrated (4, 9) before the discovery of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (34), have been shown subsequently to be due to the functional interactions of the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger (NCX1) and the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (16, 17, 32). There is ample evidence to indicate that digitalis-induced partial inhibition of cardiac Na\textsuperscript{+}/K\textsuperscript{+}-ATPase leading to a modest rise in intracellular Na\textsuperscript{+} concentration is sufficient to affect the robust cardiac Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger, causing a significant increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]), and cardiac contractility (16, 32). This positive inotropic action of digitalis has long been considered to be a basis of the major classical use of these drugs for the treatment of heart failure (9, 14, 36).

In more recent years, it has been realized that when digitalis drugs bind to the Na\textsuperscript{+}/K\textsuperscript{+}-ATPases of various intact cell types, including cardiac myocytes, they also initiate or alter the interactions of the enzyme with neighboring membrane proteins, activating multiple cell signaling pathways that have highly cell-specific and growth-related consequences (27, 40). The two parallel signaling sequences that are activated by positive inotropic but nontoxic digitalis concentrations in cardiac myocytes are the EGFR-Src/Ras/Raf/ERK pathway and the PI3K1A/PDK/Akt pathway (21, 40). These digitalis-activated pathways lead to myocyte hypertrophy that seems to be akin to physiological hypertrophy (21).

Although the positive inotropic and cell signaling actions of digitalis drugs are both mediated through the cardiac myocyte Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, the nature of the relationship, if any, between these two drug effects on the heart has not been clear. The primary goal of this work was to clarify this issue. Since digitalis was expected not to affect contractility in myocytes devoid of NCX1 (31), we used the hearts and myocytes from wild-type (Wt) and NCX1 cardiac-specific knockout (KO) mice to compare ouabain’s positive inotropic effect and its effects on cell signaling and hypertrophic growth. Our findings suggest that while the exchanger is essential to ouabain’s positive inotropic effect and some of ouabain’s signaling effects, the exchanger is not involved in the drug’s signaling effects that lead to myocyte hypertrophy and are likely to be beneficial to the heart.

MATERIALS AND METHODS

Mice. All research on mice was done according to procedures and guidelines of the National Institutes of Health, and the protocols were approved by the Institutional Animal Care and Use Committee of the University of Toledo, College of Medicine and Life Sciences.

Isolated perfused hearts. Male KO mice or Wt littermates (8–12 wk old) were anesthetized with pentobarbital sodium (150 mg/kg ip) and heparinized (1,000 U/kg). The perfusion protocol was a modification of those we had used before for the rat heart (25, 28) with adjustments specific to the use of the mouse heart (37). Briefly, hearts were rapidly removed and mounted on a nonrecirculating Langendorff apparatus and perfused with Krebs-Henseleit (KH) buffer containing (in mM) 118.0 NaCl, 4.0 KCl, 1.65 CaCl\textsubscript{2}, 1.3 KH\textsubscript{2}PO\textsubscript{4}, 1.2 MgSO\textsubscript{4},...
0.3 ethylene glycol bis(2-aminoethyl)ether)-N,N',N''-tetraacetic acid, 25 NaHCO₃, and 11 d-glucose. The KH buffer (pH 7.4) was continuously oxygenated with a mixture of O₂ (95%) and CO₂ (5%), and the temperature was maintained at 37°C with a temperature-controlled circulating water bath. The KH buffer was delivered at a constant flow rate of 3–5 ml/min, resulting in an initial coronary perfusion pressure (CPP) of ~100 mmHg at the aortic cannula. End-diastolic pressure (EDP), the rate of pressure development (+dP/dt), and the rate of relaxation (−dP/dt) were measured via a water-filled balloon inserted in the left ventricle. The EDP was adjusted initially to ~5 mmHg, and the hearts were paced at 400 beats/min (0.5 ms, 4 V) with bipolar electrodes attached to the left ventricle using a Grass SD9 stimulator. After 30 min of stabilization, Wt and KO hearts were exposed to KH for 12 min (control), or to KH for 8 min followed by 4 min of treatment with 50 μM ouabain. The pressure signals were augmented via a Grass CP12 AC/DC strain gage amplifier. PowerLab and LabChart software (AD Instruments, Sydney, Australia) were used for data acquisition and analysis, respectively.

Adult mouse cardiac myocytes. Isolation and culture of the myocytes were performed according to previously described procedures (26). Briefly, mice were heparinized (5,000 U/kg) and anesthetized with pentobarbital sodium (100–140 mg/kg) and anesthesized with pentobarbital sodium (100–140 mg/kg) via intraperitoneal injection. Hearts were excised and cannulated via the aorta and connected to a modified Langendorff apparatus, perfused for 5 min with a Ca²⁺-free solution, and then with a solution of 1 mg/ml Type II collagenase (Worthington Biochemical) for 10–20 min. The yield of myocytes per heart was over one million, with 70–90% being rod-shaped cells. Myocytes were plated at the density of 25,000/ml on laminin (2 μg/cm²)-coated culture plates in Modified Eagle’s Medium (MEM) supplemented with 2 mM ATP, 2 mM glucose, 10% fetal bovine serum, 10 mM 2,3-butanedione monoxime (BDM), and 100 μM penicillin (pH 7.0) at 37°C in a 2% CO₂ incubator for 1 h. Cell bovine serum, 10 mM 2,3-butanedione monoxime (BDM), and 100 μM penicillin (pH 7.0) at 37°C in a 2% CO₂ incubator for 1 h. Cell attachment rate was about 80% per well. For cell signaling experiments, myocytes were cultured in MEM containing BDM and 0.1% BSA overnight. Cells were then transferred to the same medium without BDM before the indicated treatments and assays.

Immunoblot analysis. Myocytes were lysed using RIPA buffer and analyzed as described before (20). Lysate samples containing equal amounts of protein were subjected to 8 or 4–12% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences), and probed with appropriate antibodies by standard procedures. The immunoreactive bands were detected using enhanced chemiluminescence (Pierce). Images were scanned with a densitometer. For quantitative immunoassays, 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 (20).

Assays of ERK, Akt, and Na⁺-/K⁺-ATPase subunits. Activation of ERK1/2 was determined by quantitative immunoblots as indicated above, using a monoclonal anti-phospho-ERK1/2 antibody that detects ERK1/2 only when they are phosphorylated at Thr202 and Tyr204. The same blots were stripped and probed with a polyclonal antibody recognizing the total amount of ERK1/2 to account for equal loading. Activation of Akt was determined by probing phosphorylated Akt (Ser473) and followed by total Akt. Immunoblots were then quantified and the ratios of p-ERK1/2 to ERK1/2, and p-Akt to Akt band density were calculated. The ratio of band density of each group was then divided by untreated controls to calculate the fold increase/decrease from multiple independent experiments. For the proteins of NCX1 and Na⁺-/K⁺-ATPase subunits, GAPDH was used as a loading control.

Primary antibodies and their sources were as follows: RDI Research Diagnostics, rabbit anti-Na⁺/Ca²⁺ exchanger; BD Transduction Laboratories, anti-Pi3K p85; Cell Signaling Technology, rabbit anti-phospho 473-Akt and anti-Akt; Developmental Studies Hybridoma Bank, University of Iowa: Na⁺-/K⁺-ATPase α1 (c6f6); ABR, Na⁺/K⁺-ATPase α2; Millipore, Na⁺/K⁺ ATPase β1; Santa Cruz, anti-phospho-ERK and ERK. Secondary antibodies goat anti-rabbit IgG-horseradish peroxidase (HRP) and goat anti-mouse IgG-HRP were purchased from Santa Cruz.

Assays for Pi3K/Akt enzyme activity. This was conducted by the same procedure as we previously described in detail (21). Briefly, myocyte proteins dissolved in RIPA buffer were incubated with anti-Pi3K p85ε antibody, the immune complex was collected, and equal amounts of Pi3K, as determined by quantitative immunoassay of p85 subunits, were assayed for lipid kinase activity by incubation with phosphatidylinositol and [γ-32P]ATP. The resulting 32P-labeled lipids were then separated on TLC plates, and the relevant lipid product of Pi3K; i.e., PIP3, was quantified by a phosphomager.

Measurement of protein synthesis rate. This was conducted using [3H]leucine incorporation into cell proteins as described previously (6). Briefly, adult myocytes were prepared and cultured in medium with BDM in 12-well culture plates and serum deprived for 4 h as indicated above before ligand additions in the presence of 1 μCi of labeled amino acid, and incubation for 12 h. Cells were precipitated in 5% trichloracetic acid after PBS wash. Acid-precipitable counts were dissolved in 0.2 N NaOH/0.1% SDS and counted in a scintillation counter.

Real-time quantitative RT-PCR. Total RNA was isolated with the RNeasy mini kit (Qiagen) with column DNase digestion with the RNase-free DNase set (Qiagen) according to the manufacturer’s instructions. High-capacity cDNA reverse transcription kit (AB Applied Biosystems) was used for RT-PCR. Real-time PCR was performed by the SYBR Green method with an Applied Biosystems 7500 Fast Real-Time PCR System. All samples were run in triplicate. In all cases, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used for data standardization and normalization. Gene expression levels and fold change comparisons were assessed using the ΔΔCt (cycle threshold) and ΔCt, respectively. Primers (forward and reverse, 5’ → 3’) were ANP: TCG TCT TGG CCT TTT GCC T and TCC AGG TGG TCT AGC AGG TTT BNP: AAG TCC TAG CCA GTC TCC AGA and GAG CTG CTG CGT GGC CAT TTC; β-MHC: ATG TCG CCG ACC TGG GAA G and CCT CGG GTT AGC AGA GAC ATC A; and GAPDH: CAT GCC CTT CCG TGT TCA and CCT GCT TCA CCA TCT TGA T. [Ca²⁺]i measurement. To measure [Ca²⁺], changes, adult myocytes were freshly isolated in the presence of BDM as described above and used within 5 h of isolation. Suspended cells were then washed with Hank’s Balanced Salt Solution supplemented with 1 mg/ml BSA and 25 mM HEPES, pH 7.4. Cells were incubated with 2 μM fura-2 AM for 20 min at room temperature. Following loading, myocytes were placed in a slotted bath chamber on the stage of a Nikon Eclipse TE-2000-s microscope for recording. Only quiescent, rod-shaped myocytes with well-defined edges were chosen and stimulated at 20 V, 0.2 Hz, for 5-ms duration using A-M Systems Isolated Pulse Stimulator (model 2100). Cells were perfused with bath solutions containing different concentrations of ouabain using a pressure-controlled perfusion system (Warner V6 six channel valve controller). Fluorescence was alternatively excited at 340 nm and 380 nm and emitted fluorescence measured at 510 nm. Fluorescence images were acquired and analyzed using Till Photonics Polychrome IV high-speed monochromator-based imaging system as previously described.
Each measurement was obtained from a single myocyte per coverslip, and data from multiple coverslips were pooled. Statistical analysis of data. Data are expressed as means ± SE. All analyses were performed on SPSS (SPSS, Chicago, IL, Release 17.0). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Positive inotropic effects of ouabain in control and NCX1 knockout hearts. NCX1 was shown before to be necessary for ouabain’s effect on the contractility of the mouse embryonic heart tubes of animals with global KO of the exchanger (31). To see if NCX1 is also essential for this ouabain action in the adult mouse heart, we used a mouse with the cardiac-specific KO of NCX1 (10) and compared the effects of ouabain on isolated Langendorff preparations of the Wt and the KO hearts. As shown in Table 1, basal $+dP/dt$ was decreased by ~20 percent in the KO hearts compared with littermate Wt hearts, indicative of a moderate decrease in contractile function in the KO hearts as previously noted in vivo and in Langendorff preparations of the young mouse heart (31).

**Fig. 1.** Comparative effects of ouabain on the contractilities of the wild-type (Wt) and Na$^+$/Ca$^{2+}$ exchanger (NCX1) knockout (KO) hearts. Top: representative $dP/dt$ recordings in basal conditions (8 min) prior to perfusion with 50 μM ouabain (oua, 4 min) in Wt and NCX1 KO hearts. Bottom: $+dP/dt$ from 6–7 hearts/group. Shown are values ± SE. **$P < 0.01$ vs. untreated. □, Untreated hearts (12 min KH buffer); ■, ouabain-treated hearts (8 min KH buffer followed by 4 min ouabain, 50 μM).**

**Fig. 2.** Expressions of NCX1 (A) and the major subunits of Na$^+$/K$^+$-ATPase (B) in adult myocytes isolated from Wt and NCX1 KO hearts. Myocyte isolation and protein immunoassays were done as indicated in METHODS. GAPDH was used as a loading control. $n = 3–4$, *$P < 0.05$ vs. Wt.
perfused hearts (10, 11). Positive inotropy caused by 50 μM ouabain that was clearly evident in control hearts was greatly reduced in the NCX1 KO hearts. In the KO hearts, ouabain failed to cause significant changes in +dP/dt compared with significant ouabain-induced changes in controls (Fig. 1).

Immunoblotting of NCX1 showed that its level in myocytes of the KO hearts was ~10 percent of that in control myocytes (Fig. 2A), in agreement with previous assays of the exchanger in the whole hearts of these mice (10). It has been established that in these KO hearts, as in the case of many other cardiac-specific ablations, about 80–90 percent of myocytes are devoid of NCX1, but the remaining cells have normal levels (10). Immunoblotting of the subunits of Na+/K+-ATPase indicated similar levels of α1 and β1 subunits in Wt and KO myocytes, but a lower level of α2 subunit in the KO myocytes (Fig. 2B). In mouse cardiac myocytes, α1 subunit constitutes about 70–80% of the total α subunit content (7).

Since ouabain’s effect on contractility is accompanied by an increase in [Ca2+], we externally paced myocytes isolated from the control and the KO hearts, and compared the effects of ouabain on Ca2+ transient amplitudes. Resting ratio levels were not different in control and KO cells (Fig. 3A), in agreement with previous observations (9). While ouabain clearly increased the transient amplitudes in the control cells as expected, it had no significant effects on the transient amplitudes of the KO cells (Fig. 3, B and C).

**Ouabain-activated signaling pathways in control and NCX1 knockout myocytes.** Our previous studies showed ouabain activations of the EGFR-Src/Ras/Raf/ERK and the PI3K/AKT pathways in rat cardiac myocytes (21). Here we used the adult myocytes of the mouse hearts to examine ouabain effects on these pathways. Significant dose-dependent activations of ERK 1/2 by ouabain were noted in Wt myocytes, but not in those of the KO myocytes (Fig. 4A and B). Comparable activations by endothelin-1 (ET-1) in the control and KO myocytes (Fig. 4C) demonstrate that suppression of ouabain-induced ERK 1/2 activation in the KO myocytes was not due to failure of ERKs to respond to extracellular stimuli in these

![Graphs showing effects of ouabain on Ca2+ transient](http://ajpheart.physiology.org/)

**Fig. 3.** Effects of ouabain (oua) on Ca2+ transient in paced adult myocytes from Wt and NCX1 KO hearts. Myocytes were loaded with fura-2. Experiments were conducted as described in METHODS using 10 μM ouabain. A: average resting ratio value from Wt and KO myocytes. B: representative traces of calcium transient induced by pacing at 0.2 Hz. C: quantitative data of average peak amplitudes of evoked Ca2+ transient relative to control values. Wt (n = 9) and KO (n = 10). *
P < 0.05 vs. Wt control.
cells. Ouabain-induced activations of Akt (Fig. 5) and PI3K/Akt (Fig. 6) were similar in Wt and KO myocytes.

Ouabain-induced hypertrophy in control and NCX1 knock-out myocytes. Our previous studies on rat cardiac myocytes suggested that ouabain-induced hypertrophy is linked to the activation of PI3K/Akt pathway and not due to the ouabain activation of EGFR-Src/ERK pathway (21). In view of this and the present findings illustrated in Figs. 4–6, it was important to compare ouabain’s effects on hypertrophic growth of the isolated Wt and the KO myocytes. We used increase in the rate of protein synthesis as a measure of hypertrophy. It is well-established that cardiac myocyte hypertrophy is due to an increased rate of protein synthesis (6, 8, 23, 33); and we have shown that ouabain-induced hypertrophy of cardiac myocytes is not accompanied by an increased number of cells (21, 27). The results of the experiments summarized in Fig. 7 clearly showed that ouabain caused similar stimulations of protein synthesis in both sets of myocytes. As a positive control, another known stimulus of hypertrophy, ET-1, also increased protein synthesis rates in both sets of myocytes (Fig. 7).

To examine the role of the PI3K/Akt and EGFR-Src/ERK pathways in the ouabain-induced increase in protein synthesis in Wt and KO myocytes, we utilized two commonly used inhibitors of the ouabain-activated signaling pathways: wortmannin, an irreversible inhibitor of PI3Ks, and U0126, an MEK inhibitor that prevents the activations of ERKs. Because
of the known potential effects of PI3Ks and ERKs on cardiac myocyte hypertrophy (2), it was necessary to assess the effects of wortmannin and U0126 alone in addition to the effect of each inhibitor in combination with ouabain. The findings indicate that Wortmannin alone significantly reduced protein synthesis rate in both Wt and KO myocytes, and that ouabain did not stimulate protein synthesis in the presence of wortmannin (Fig. 8). In contrast, U0126 alone had no significant effect on protein synthesis, but it allowed ouabain to stimulate protein synthesis in both Wt and KO myocytes (Fig. 8).

Because pathological cardiac myocyte hypertrophy and remodeling have been associated with various patterns of marker gene expression (2, 22, 38), we assessed ouabain-induced changes in mRNA levels of brain natriuretic peptide (BNP), atrial natriuretic peptide (ANP), and /H9252-myosin heavy chain (/H9252-MHC), three genes whose discordant upregulations are often noted in stressed hearts (38). Under the same conditions where ouabain increased myocyte protein synthesis (Figs. 7 and 8), it had no significant effects on the mRNA levels of any of the marker genes either in the Wt or the KO myocytes (Fig. 9). In contrast, ET-1 significantly upregulated BNP mRNA levels in both Wt and KO myocytes, but had no apparent effects on the other two marker genes (Fig. 9).

**DISCUSSION**

*Necessity of NCX1 for ouabain’s positive inotropic effect.* In previous studies on embryonic heart tubes obtained from mice with global KO of NCX1, the necessity of the presence of the exchanger for the positive inotropic action of ouabain was indicated (31). Because this global KO was embryonic lethal, however, it was not possible to extend the same conclusion to the adult heart. In the present studies, therefore, we reexamined the issue using the more recently developed mouse with cardiac-specific KO of the exchanger which lives to adulthood with nearly normal cardiac function due to adaptive changes in the transsarcolemmal fluxes of the L-type Ca\(^{2+}\) channels (10, 29, 30). There are no significant changes in the expression of numerous myocardial proteins involved in excitation-contraction coupling due to this KO (10), and contractile and metabolic responses to isoproterenol are comparable in the control and the KO hearts (10, 11). Our present findings demonstrate that ouabain-induced positive inotropy in the adult heart and the adult cardiac myocytes (Table 1 and Figs. 1–3) also requires the presence of NCX1. In conjunction with numerous other types of previously accumulated evidence (1, 16, 17, 32), we now consider it well-established that the positive inotropic effects of ouabain and related cardiac glycoside drugs require the functional interaction of NCX1 and the cardiac Na\(^+/K^+\)-ATPase (Fig. 10, left).

*Irrelevance of ouabain-induced inotropy to the drug’s hypertrophic effect.* Pioneering studies on cardiac myocyte hypertrophic growth had established that while contraction per se...
A n alone.

\[ \text{MHC; H9252/H9252-myosin heavy chain (Fig. 9).} \]

Lack of effect of ouabain on the expression of hypertrophic marker genes [atrial natriuretic peptide (ANP; A), brain natriuretic peptide (BNP; B), and \( \beta \)-myosin heavy chain (\( \beta \)-MHC; C)] in myocytes of Wt and NCX1 KO hearts. Cultured myocytes were incubated with the indicated ouabain concentrations and ET-1 (positive control) for 6 h. Total RNA was isolated and the indicated marker RNAs were analyzed by real time RT-PCR. \( n = 3–4 \), \(* P < 0.05\) vs. control.

DISCUSSION.

Increases cell size, hypertrophy induced by some stimuli, e.g., adrenergic agonists, may be independent of their enhancement of contractility (8, 23, 33). Upon discovery of ouabain’s hypertrophic effect on myocytes, which was accompanied by the rise in \([\text{Ca}^{2+}]\), the drug’s hypertrophic effect persists (Fig. 7), thus clearly relating the ouabain-activated signaling pathways that are independent of the rise in \([\text{Ca}^{2+}]\), in the process leading to hypertrophy.

**Linkage of PI3K-Akt pathway to ouabain-induced hypertrophy.**

To date, two parallel ouabain-activated cell signaling pathways have been found in cardiac myocytes, i.e., the EGFR-Src/Ras/Mek/Erk and the PI3K/Akt (21, 40). The former has been shown by our previous studies (20, 24, 25) to be
dependent on ouabain-induced increase in \([\text{Ca}^{2+}]/\text{K}^+\)-ATPase, and as depicted in Fig. 10. Therefore, it is not surprising that ouabain-induced activations of ERK1/2 are greatly suppressed in myocytes lacking NCX1 (Fig. 4). Since ouabain stimulates PI3K IA, Akt, and protein synthesis in both Wt and KO myocytes (Figs. 5–7), it seems that only the activation of PI3KIA/PDK/Akt pathway is independent of \([\text{Ca}^{2+}]/\text{K}^+\) and needed for ouabain-induced hypertrophy (Fig. 10). This conclusion is further strengthened by our findings of Fig. 8, indicating that ouabain’s effect on protein synthesis persists in the presence of a MEK/ERK inhibitor but not in the presence of a PI3K inhibitor.

The apparent irrelevance of the ouabain activation of EGFR-Src/Ras/Raf/ERK pathway to ouabain-induced hypertrophy (Fig. 10) is reminiscent of the noted redundancy of ERK activation to the actions of other hypertrophic stimuli (2, 13). It remains to be seen if the extensively studied effects of ouabain on the pathway that begins with the coactivation of EGFR and Src have a role, if any, in the regulation of ouabain-initiated myocyte hypertrophy that is mediated through the PI3KIA/Akt pathway.

Although the scheme of Fig. 10 is a reasonable summary of the conclusions of the present studies, it is appropriate to discuss briefly one of its obvious limitations. In the depicted initial interaction of digitalis with Na\(^+\)/K\(^+\)-ATPase, we have not included the well-known existence of the multiple isoforms (\(\alpha_1\) and \(\alpha_2\)) of the enzyme in adult rodent myocytes, the isoforms’ different digitalis sensitivities, and the suggested but not established linkage of different digitalis-activated cell signaling pathways to different cardiac isoforms (e.g., 20, 21). There is also an extensive literature (7 and references cited therein) to suggest that cardiac isoforms may be differentially localized, and modulate \([\text{Ca}^{2+}]/\text{K}^+]\), differently in the absence or the presence of digitalis. With the expected future progress on the clarification of the different roles of the isoforms, refinements of the schemes of Fig. 10 will certainly be required.

Also related to the above complexities are our findings of Fig. 2, indicating that the reduced expression of NCX1 seems to be accompanied by a lowered level of the \(\alpha_2\) isofrom, but not that of the \(\alpha_1\) isofrom. The potential significance of this finding must await the further resolution of the ambiguities on the special roles of the isoforms in the regulation of digitalis-induced contractility, and the determination of whether or not the changed expression level of either isofrom affects digitalis-induced signaling that leads to myocyte hypertrophy through the PI3KIA pathway.

On the nature of ouabain-induced hypertrophy. There is increasing evidence to indicate the existence of distinct molecular mechanisms of physiological and pathological cardiac hypertrophy, and different roles of PI3K isoforms in such mechanisms (2, 6, 22, 39). In our previous studies (21) showing that ouabain activates cardiac myocyte PI3KIA but not PI3KIB, we suggested the superficial similarity of ouabain-induced hypertrophy to physiological hypertrophy. Further support for this suggestion is also provided by our present findings on the regulations of several fetal genes by ouabain and ET-1 (Fig. 9). ET-1 is known to cause pathological hypertrophy of myocytes through the activation of the Gq-responsive PI3KIB (2, 39). Our findings on the discordant upregulations of the fetal genes by ET-1 (Fig. 9) are in keeping with previous findings on such upregulations of these genes by ET-1 and other stimuli of pathological hypertrophy (2, 38). Therefore, in spite of the recognized limitations of the studies on the changing patterns of fetal gene programs (2), the fact that ouabain, in contrast to ET-1 but like exercise (2, 39), has no apparent effect on the fetal genes (Fig. 9) provides further support to the hypothesis that ouabain-induced hypertrophy may have some similarity to physiological hypertrophy. Since experimentally induced physiological hypertrophy through the activation of PI3KIA is indeed capable of antagonizing or reversing the consequences of pathological hypertrophy (2, 3, 18, 22), more direct and rigorous testing of the potential benefits of digitalis treatment on induced pathological hypertrophy seems to be appropriate. Such studies remain to be done.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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