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# Signal-transducing function of $Na^+$-$K^+$-ATPase is essential for ouabain's effect on $[Ca^{2+}]_i$ in rat cardiac myocytes

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**Tian, Jiang, Xiaohua Gong, and Zijian Xie.** Signal transducing function of $Na^+$-$K^+$-ATPase is essential for ouabain's effect on $[Ca^{2+}]_i$ in rat cardiac myocytes. *Am J Physiol Heart Circ Physiol* 281: H1899–H1907, 2001.—We showed before that $Na^+$-$K^+$-ATPase is also a signal transducer in neonatal rat cardiac myocytes. Binding of ouabain to the enzyme activates multiple signal pathways that regulate cell growth. The aims of this work were to extend such studies to adult cardiac myocytes and to determine whether the signal-transducing function of $Na^+$/$K^+$-ATPase regulates the well-known effects of ouabain on intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). In adult myocytes, ouabain activated protein tyrosine phosphorylation and p42/44 mitogen-activated protein kinases (MAPKs), increased production of reactive oxygen species (ROS), and raised both systolic and diastolic $[Ca^{2+}]_i$. Pretreatment of myocytes with several Src kinase inhibitors, or overexpression of a dominant negative Ras, antagonized ouabain-induced activation of MAPKs and increases in $[Ca^{2+}]_i$. Treatment with PD-98059 (a MAPK kinase inhibitor) or overexpression of a dominant negative MAPK kinase 1 also ablated the effect of ouabain on MAPKs and $[Ca^{2+}]_i$. *N*-acetyl-cysteine, which blocks the effect of ouabain on ROS, did not prevent the ouabain-induced rise in $[Ca^{2+}]_i$. Clearly, the activation of the Ras/MAPK cascade, but not ROS generation, is necessary for ouabain-induced increases in $[Ca^{2+}]_i$ in rat cardiac myocytes.

signal transduction

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$Na^+$-$K^+$-ATPASE is an energy-transducing ion pump in most mammalian cells (20, 29). It carries out the active transport of $Na^+$ and $K^+$ across the plasma membrane. This enzyme also serves as a functional receptor for digitalis compounds such as ouabain (6, 20, 27). At nontoxic concentrations, ouabain causes a partial inhibition of the ion-pumping function of cardiac $Na^+$-$K^+$-ATPase. This can lead to a small increase in intracellular $Na^+$ concentration, which in turn raises intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) through the $Na^+$/$Ca^{2+}$ exchanger (2, 3, 18).

Recently, studies (33, 34) from our laboratories have revealed several previously unknown effects of ouabain on cardiac myocytes. We found that the same nontoxic concentrations of ouabain that cause partial inhibition of $Na^+$-$K^+$-ATPase and an increase in $[Ca^{2+}]_i$ also stimulate the nonproliferative growth (hypertrophy) of myocytes and regulate the transcription of a number of growth-related genes (13, 24). These ouabain effects involve the activation of multiple signal transduction pathways, including the stimulation of Src kinase and tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) and other proteins, followed by the activation of Ras (10, 16). Downstream from Ras, ouabain stimulates at least two important signal pathways. One of the pathways leads to the activation of mitogen-activated protein kinases (MAPKs), and the other causes increased production of reactive oxygen species (ROS) by mitochondria (16, 21). Both pathways play an important role in ouabain regulation of cell growth and gene expression in neonatal cardiac myocytes (35). Interestingly, several of the early signaling events, including the stimulation of protein tyrosine kinases and production of ROS, are independent of ouabain-induced changes in intracellular ion concentrations (21). These findings led us to propose that there are at least two distinct pools of $Na^+$-$K^+$-ATPase in neonatal cardiac myocytes (see Fig. 11 of Ref. 21): One pool exhibits its classic function as an energy-transducing ion pump, and the other is involved in signal transduction (33). Our prior studies (13, 24, 35) also indicated that the ion-pumping function of $Na^+$-$K^+$-ATPase contributed significantly to the ouabain-mediated transcriptional regulation of cardiac genes because the effects of ouabain on gene expression depended on increases in both ROS and $[Ca^{2+}]_i$. Furthermore, because increases in $[Ca^{2+}]_i$ are not required for some of the ouabain-induced early signaling events, including activation of protein tyrosine kinases and increased production of ROS, it is clear that $[Ca^{2+}]_i$ cooperates with increased ROS to regulate distal events and cross-talk among the pathways that are important for ouabain regulation of the genes (21). These findings prompted us to ask whether the signal-transducing function of the enzyme can also contribute to ouabain-induced increases in $[Ca^{2+}]_i$ in cardiac myocytes. Early studies (6, 22) of others had already suggested that the effects of ouabain on $[Ca^{2+}]_i$ may involve not only the $Na^+$/$Ca^{2+}$ exchanger but also other

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membrane transporters that appear to be regulated by ouabain-activated protein kinases. Thus it was logical to ask if the protein kinases that are activated by ouabain through the signal-transducing function of $Na^+$-$K^+$-ATPase may be involved in the regulation of $[Ca^{2+}]_i$. In the studies presented here, we established first that the signal-transducing function of $Na^+$-$K^+$-ATPase previously observed in neonatal cardiac myocytes also exists in adult cardiac myocytes. We then explored the relation of these signal-transducing functions to the effects of ouabain on $[Ca^{2+}]_i$.

## MATERIALS AND METHODS

*Materials.* Chemicals of the highest purity were purchased from Sigma (St. Louis, MO). Collagenase type II was from Worthington (Freehold, NJ). Indo 1-AM, fura 2-AM, and 5-(and 6-)chloromethyl-2′,7′-dichlorofluorescein (CM-DCFH) diacetate were obtained from Molecular Probes (Eugene, OR). Genistein, herbimycin A, and PP2 were purchased from Calbiochem (San Diego, CA). The antibodies used and their sources were as follows: anti-phosphotyrosine monoclonal antibody (PY99), MAPK polyclonal antibodies, and goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified anti-active MAPK antibodies were purchased from Promega (Madison, WI). Goat anti-mouse or anti-rabbit secondary antibodies were purchased from Pierce (Rockford, IL). The Optitran nitrocellulose membranes used for Western blotting were obtained from Schleicher and Schuell (Keene, NH).

*Cell preparation and culture.* The same protocol was used to prepare $Ca^{2+}$-tolerant adult rat ventricular myocytes as described in our previous work (36). In brief, Sprague-Dawley rats weighing between 250 and 300 g were anesthetized with pentobarbital sodium (60 mg/kg ip), and the hearts were rapidly removed. The heart was then attached to an aortic cannula and retrograde perfused for 15 min with Joklik medium to wash out the blood, followed by 5-min perfusion with a nominally $Ca^{2+}$-free Joklik's medium supplemented with 20 mM creatine and 60 mM taurine. The heart was then perfused with the same medium containing 0.2 mg/ml collagenase Type II and 50 μM $CaCl_2$, and the perfusate was recirculated until the heart became soft and flaccid. Myocytes were dissociated from the left ventricle and harvested. This method of isolation produced a good yield of rod-shaped (70–80%) myocytes. To culture adult rat cardiac myocytes, cells were suspended in serum-free M199 and plated onto laminin-coated coverslips as previously described (28). The ionic composition of M199 was as follows (in mM): 135 $Na^+$, 5.4 $K^+$, 0.8 $Mg^{2+}$, and 1.8 $Ca^{2+}$. Medium was changed 2 h postplating. Over 95% of myocytes were quiescent, and they were used for the experiments after an overnight culture.

*Fluorescence microscopic measurements of $[Ca^{2+}]_i$ and ROS.* Myocytes cultured on coverslips were perfused. To elicit myocyte contraction, cells were field stimulated with platinum electrodes at a frequency of 0.5 Hz and a duration of 5 ms using a Grass S11 dual-output digit stimulator (Quincy, MA). In general, myocytes were paced for 5 min to stabilize calcium transients before various treatments were initiated. Control experiments showed that once calcium transients stabilized, the cells could be used for at least 30 min under our experimental conditions. $[Ca^{2+}]_i$ was measured by either fura 2 or indo 1 as previously described (21). Myocytes were loaded with 10 μM indo 1-AM for 30 min. Indo 1 fluorescence was recorded using a microscope-based fluorescence system (Photon Technology; Monmouth Junction, NJ). The probe was excited at 365 nm, and fluorescence emitted at 405 and 485 nm was recorded at 60 Hz in real time. $[Ca^{2+}]_i$ was calculated based on the fluorescence ratio and the $Ca^{2+}$calibration curve (21). Because intracellular pH affects $Ca^{2+}$ binding to indo 1 and fura 2, the effects of ouabain on intracellular pH were measured in myocytes using 2′,7′-bis-(2-carboxyethyl)-5-(6-)carboxyfluorescein as a probe. As previously reported (5a), we found that nontoxic concentrations of ouabain (up to 100 μM) caused no detectable changes in intracellular pH in these myocytes (data not shown). To verify the $Ca^{2+}$ results of indo 1 experiments, some of the experiments were repeated in fura 2-loaded myocytes. Fura 2 fluorescence was recorded at a speed of 30 Hz using an Attofluor imaging system (Atto Instruments) at an excitation wavelength of 340/380 nm and emission wavelength of 505 nm (21). Intracellular ROS production was measured in cells loaded with 10 μM of reduced 5-(and 6-)chloromethyl-2′,7′-dichlorofluorescein (CM-DCF) diacetate, as previously described (21, 35). Under each experimental condition, ~15 single myocytes from three independent preparations were imaged with an Attofluor imaging system (Atto Instruments), and CM-DCF fluorescence was measured at an excitation wavelength of 480 nm and emission wavelength of 520 nm.

*Measurement of protein phosphorylation and p42/44 MAPK activity.* Cell lysis and immunoblotting were performed as previously described (10). Briefly, after the indicated treatment, cells were washed with 5 ml of ice-cold PBS and lysed in 200 μl of ice-cold RIPA buffer containing 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM Tris·HCl (pH 7.4). Cell lysates were centrifuged at 16,000 *g* for 10 min, and supernatants were used for Western blot analysis. To measure protein tyrosine phosphorylation and p42/44 MAPK activity, samples were separated by SDS-PAGE (60 μg/lane) and transferred to an Optitran membrane as previously described (10). The membranes were then probed with an anti-phosphotyrosine monoclonal antibody or anti-active MAPK polyclonal antibody. The anti-active MAPK polyclonal antibody was then stripped, and the membrane was reprobed with a polyclonal antibody that recognizes the total amount of MAPK to account for equal loading, as previously reported (10). The secondary antibodies were conjugated to horseradish peroxidase; hence, the immunoreactive bands were developed using chemiluminescence (Pierce, Rockford, IL).

*Preparation of replication-defective adenoviruses and adenovirus infection of cardiac myocytes.* Replication-defective adenoviruses expressing either a dominant negative $Asn^{17}$ Ras or $Ala^{221}$ MAPK kinase 1 (MEK1) were prepared and used for the infection of myocytes as described before (14, 16). An identical virus containing the β-galactosidase (β-Gal) gene instead of $Asn^{17}$ Ras was used as the control (16). Control Western blot analysis showed that, like in neonatal myocytes, 12-h infection with $Asn^{17}$ Ras caused a significant increase (2.6-fold over control) in Ras protein in cardiac myocytes. An increase in MEK1 similar to that of Ras was observed in myocytes transduced with $Ala^{221}$ MEK1 virus.

*Analysis of data.* Data are given as means ± SE. Statistical analysis was performed using the Student's *t*-test, and significance was accepted at $P < 0.05$. Each presented immunoblot is representative of the similar results from at least three separate experiments.



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## RESULTS

*Time- and dose-dependent effects of ouabain on $[Ca^{2+}]_i$ in adult rat cardiac myocytes.* Binding of ouabain to $Na^+$-$K^+$-ATPase inhibits the ion-pumping function of the enzyme. Our previous studies (24) measured the time-averaged changes in $[Ca^{2+}]_i$ in response to nontoxic concentrations of ouabain in neonatal cardiac myocytes. The experiments shown in Figs. 1 and 2 show the effects of ouabain on both systolic and diastolic $[Ca^{2+}]_i$ in paced adult rat cardiac myocytes. As expected, ouabain raised both systolic and diastolic $[Ca^{2+}]_i$ in these cells in a time- and dose-dependent manner, which is consistent with prior observations made in other cardiac preparations (5, 11, 31). Significant increases in $[Ca^{2+}]_i$ occurred in 1–2 min and reached steady state in 5–10 min after ouabain exposure (Fig. 1). When dose-dependent changes were examined, 10 μM ouabain caused significant changes in $[Ca^{2+}]_i$ (Fig. 2) in these myocytes. This dose-response curve correlates well with the ouabain inhibition curve on rat cardiac $Na^+$-$K^+$-ATPase (24, 36, 37). It also correlates with the ouabain curve on contractility in the rat myocardium (1, 27). It is important to note that, although ouabain significantly increased diastolic $[Ca^{2+}]_i$ at concentrations of 100 μM or lower (Fig. 1), it did not cause arrhythmic contraction and had no effect on cell viability. This is expected because ouabain at these concentrations only caused a less than twofold increase in $[Ca^{2+}]_i$. However, when cells were exposed to toxic concentrations of ouabain (e.g., > 200 μM), a large number of myocytes were $Ca^{2+}$ overloaded and underwent arrhythmic contraction and eventually contracture. Therefore, as in neonatal rat cardiac myocytes (13, 16, 35), we used 100 μM ouabain in the following experiments as the highest nontoxic concentration.

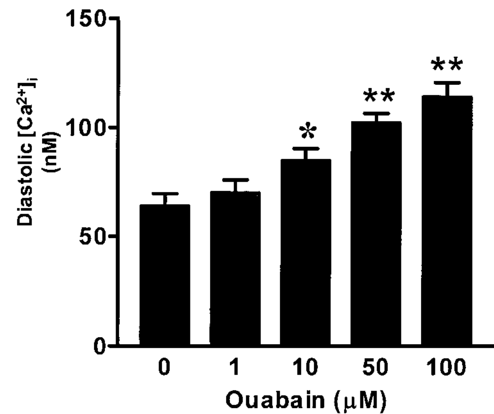


Fig. 2. Dose-response curve of the ouabain effect on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured as in Fig. 1 after the cells were exposed to different concentrations of ouabain for 10 min. Ouabain increased both systolic and diastolic $[Ca^{2+}]_i$, as in Fig. 1, in a dose-dependent manner. The effects of ouabain on diastolic $[Ca^{2+}]_i$ are presented, and values are means ± SE of 12 independent measurements from 4 experiments. $^*P < 0.05$ and $^{**}P < 0.01$ vs. control.

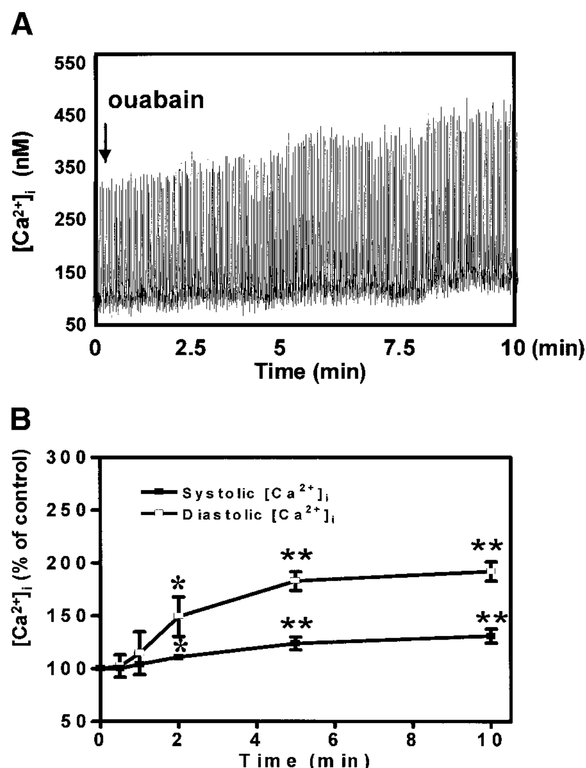


Fig. 1. Effects of ouabain on intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) as a function of time in adult rat cardiac myocytes. To measure $Ca^{2+}$ transients, cells were loaded with indo 1 and paced at 0.5 Hz. $[Ca^{2+}]_i$ was determined as described in MATERIALS AND METHODS. *A*: representative trace of $[Ca^{2+}]_i$ in a single cell. It was recorded after the cells were paced for 5 min and $Ca^{2+}$ transients were stabilized. Ouabain (100 μM) was added to the medium at the time indicated by the arrow. *B*: quantitative data of ouabain (100 μM) effects on both diastolic and systolic $[Ca^{2+}]_i$ as a function of time. $^*P < 0.05$ and $^{**}P < 0.01$ vs. control.

*$Na^+$-$K^+$-ATPase as a signal transducer in adult rat cardiac myocytes.* Because adult and neonatal cardiac myocytes are different not only in cell morphology but also in cell signaling, to examine the relationship between the signal-transducing function of $Na^+$-$K^+$-ATPase and ouabain-induced increases in $[Ca^{2+}]_i$ we first determined how ouabain regulates signal transduction pathways in adult rat cardiac myocytes. The data shown in Figs. 3 and 4 show that ouabain stimulated both protein tyrosine phosphorylation and p42/44 MAPKs in a time-dependent manner in these cells. Significant increases in tyrosine phosphorylation occurred in ~15 s (Fig. 3), whereas ouabain activated p42/44 MAPKs in <1 min (Fig. 4). The effects of ouabain on both tyrosine phosphorylation (data not shown) and p42/44 MAPKs (Fig. 4*C*) were also dose dependent, and significant stimulation was observed when myocytes were exposed to 10 μM ouabain. As in neonatal cardiac myocytes (35), ouabain also stimulated ROS production in these adult cardiac myocytes (Fig. 5). A significant increase in intracellular ROS concentration was observed after myocytes were treated with 100 μM ouabain for 30 min. As expected, the addition of 10 mM *N*-acetyl-cysteine (NAC) to the incubation medium abolished ouabain-induced increases in intracellular ROS (Fig. 5). These findings indicate that the binding of ouabain to $Na^+$-$K^+$-

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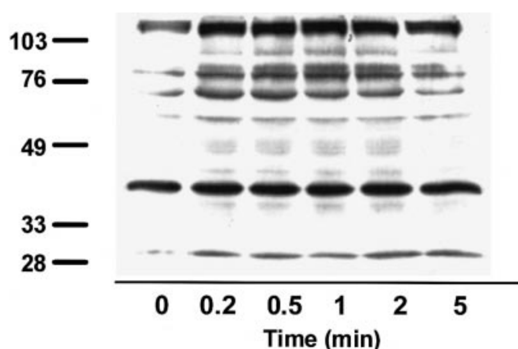


Fig. 3. Effects of ouabain on protein tyrosine phosphorylation in adult rat cardiac myocytes. After 12 h in culture, cells were treated with 100 μM ouabain for various times and assayed for protein tyrosine phosphorylation as described in MATERIALS AND METHODS. The representative Western blot of 4 independent experiments shows the effects of ouabain on protein tyrosine phosphorylation in a time-dependent way.

ATPase in adult rat cardiac myocytes is capable of initiating signaling pathways similar to those previously observed in neonatal rat cardiac myocytes (10, 21). In addition, when the temporal relationships of ouabain effects on signal transduction as well as on $[Ca^{2+}]_i$ were examined, the effects of ouabain on protein tyrosine phosphorylation appeared to precede the activation of p42/44 MAPKs and the increases in $[Ca^{2+}]_i$ in adult rat cardiac myocytes, whereas changes in both p42/44 MAPKs and $[Ca^{2+}]_i$ occur within the same time frame (Figs. 1, 3, and 4). These findings support the proposal that the binding of ouabain to $Na^+$-$K^+$-ATPase can initiate at least some signal transduction pathways independent of changes in intracellular ion concentrations (21). They also support the notion that activation of tyrosine kinases could be involved in ouabain regulation of $[Ca^{2+}]_i$ in cardiac myocytes.

In neonatal cardiac myocytes, p42/44 MAPKs are activated by ouabain through the tyrosine kinase/Ras/Raf/MEK cascade (10). The experiments shown in Fig. 6 indicate that the same pathway is also used by ouabain in adult rat cardiac myocytes. First, we found that ouabain failed to activate p42 MAPK (Fig. 6) and p44 MAPK (data not shown) in cells that were pretreated with either 100 μM genistein (a nonspecific tyrosine kinase inhibitor) or 1 μM herbimycin A (a relatively specific Src family kinase inhibitor). Second, PP2, a specific Src kinase inhibitor, also blocked the ouabain-induced activation of p42/44 MAPKs in these adult myocytes. Finally, overexpression of dominant negative $Asn^{17}$ Ras, but not β-Gal, ablated the effects of ouabain on p42/44 MAPKs (Fig. 6).

*Activation of protein tyrosine kinases and Ras is necessary for the ouabain-induced increase in $[Ca^{2+}]_i$.* The above experiments clearly demonstrated that ouabain activates protein tyrosine kinases in adult rat cardiac myocytes. To test if these kinases and other downstream signal pathways contribute to the ouabain effect on $[Ca^{2+}]_i$, we first determined the effects of genistein and herbimycin A on ouabain-induced increases in $[Ca^{2+}]_i$ in these cells. As shown in Fig. 7, both inhibitors abolished the ouabain-induced increases in diastolic $[Ca^{2+}]_i$ (Fig. 7) as well as systolic $[Ca^{2+}]_i$ (data not shown). Furthermore, when myocytes were pretreated with PP2, a Src kinase-specific inhibitor, ouabain also failed to raise $[Ca^{2+}]_i$ (Fig. 7). These findings indicate that tyrosine kinases, specifically Src kinase, not only relay the signal from ouabain binding to $Na^+$-$K^+$-ATPase to the activation of p42/44 MAPKs but are also responsible for ouabain-induced increases in $[Ca^{2+}]_i$. Because Ras relays the signal from tyrosine kinases to several downstream effectors, we assayed if

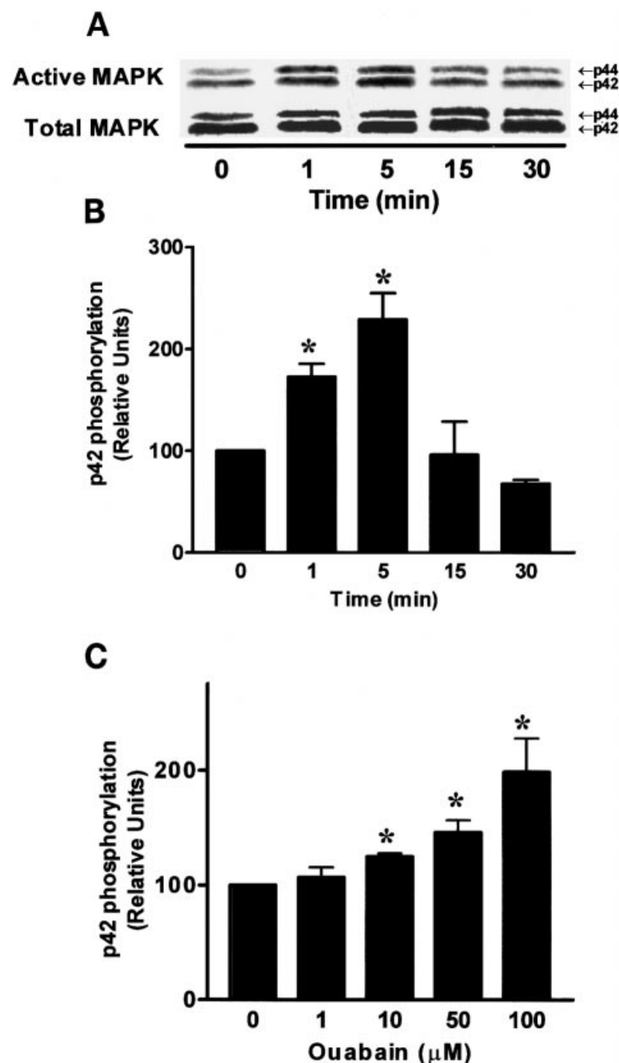


Fig. 4. Effects of ouabain on p42/44 mitogen-activated protein kinases (MAPKs). After 12 h in culture, myocytes were treated with different concentrations of ouabain for various times and assayed for active p42/44 MAPKs as described in MATERIALS AND METHODS. *A*: representative Western blot showing the time-dependent changes in levels of phosphorylated p42/44 MAPKs in response to 100 μM ouabain in adult rat cardiac myocytes. *B*: quantitative effects of 100 μM ouabain on p42 MAPK as a function of time. Values are means ± SE of 3 independent experiments. *C*: dose-dependent effects of ouabain on p42 MAPK. Values are means ± SE of 4 independent experiments. When p44 MAPK was measured, ouabain also caused a time- and dose-dependent increase in the levels of active p44 MAPK similar to that of p42 MAPK (data not shown). $*P < 0.05$ vs. control.



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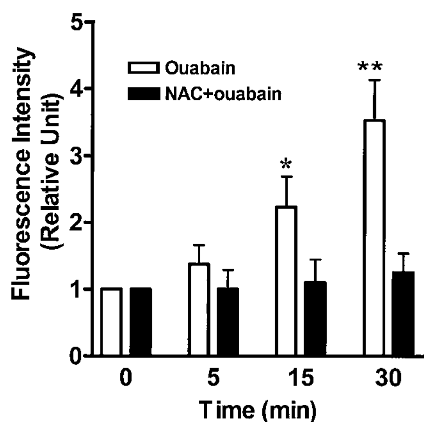


Fig. 5. Ouabain increases intracellular reactive oxygen species (ROS) in adult rat cardiac myocytes. Myocytes were loaded with 5-(and 6-)chloromethyl-2′,7′-dichlorofluorescin diacetate and treated with 100 μM ouabain for various times. Intracellular ROS were measured as described in MATERIALS AND METHODS. NAC, *N*-acetylcysteine. Values are means ± SE of 12 cells from 4 independent experiments. $^*P < 0.05$ vs. control.

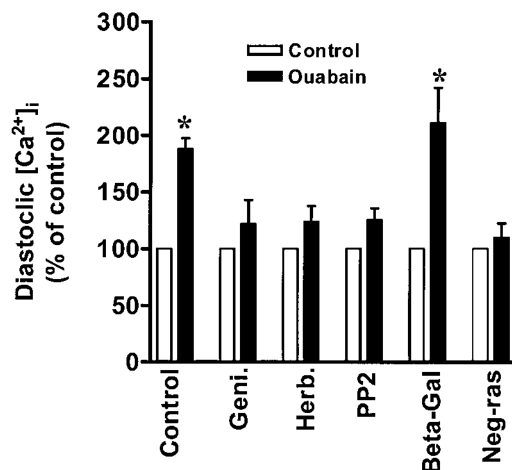


Fig. 7. Effects of inhibition of protein tyrosine phosphorylation and Ras on ouabain-induced increases in $[Ca^{2+}]_i$. The experiments were done as in Fig. 6, and $[Ca^{2+}]_i$ was measured as in Fig. 1 after the cells were exposed to 100 μM ouabain for 10 min. Values are means ± SE of 4 different experiments. $^*P < 0.01$ vs. control.

Ras is involved in the ouabain-induced increases in $[Ca^{2+}]_i$ under the same experimental conditions as in Fig. 6, in which adult cardiac myocytes were transduced with adenoviruses expressing a dominant negative $Asn^{17}$ Ras. As shown in Fig. 7, expression of $Asn^{17}$ Ras, but not β-Gal, abolished the effects of ouabain on $[Ca^{2+}]_i$. Clearly, the effects of ouabain on $[Ca^{2+}]_i$ are mediated by ouabain-activated growth pathways, requiring stimulation of protein tyrosine kinases and Ras.

*MAPKs, but not ROS, are required for the effects of ouabain on $[Ca^{2+}]_i$.* Downstream from Ras, ouabain activated p42/44 MAPKs and also increased ROS production in adult cardiac myocytes. To determine if these two pathways are involved in ouabain-induced increases in $[Ca^{2+}]_i$, adult myocytes were pretreated with the antioxidant NAC or the MEK inhibitor PD-98059. As shown in Fig. 8, PD-98059 reduced the effects of 100 μM ouabain on both systolic and diastolic $[Ca^{2+}]_i$ in a dose-dependent manner. On the other hand, as shown in Fig. 8, *B* and *C*, although 10 mM NAC seemed to repress ouabain-induced increases in $[Ca^{2+}]_i$, the effects were not statistically significant. Because the same dose of NAC abolished the effects of ouabain on ROS production, these data indicate that increases in ROS production are not a major contributor to ouabain-induced increases in $[Ca^{2+}]_i$. Together, the above findings indicate that effects of ouabain on $[Ca^{2+}]_i$ in cardiac myocytes require the activation of the p42/44 MAPK pathway.

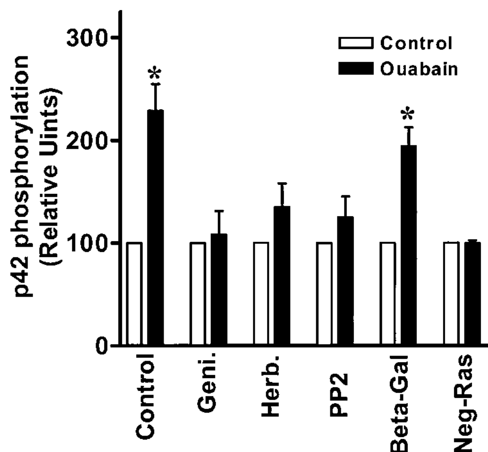


Fig. 6. Effects of inhibition of protein tyrosine kinase and Ras on ouabain-induced activation of p42 MAPKs. Myocytes were either preincubated with 100 μM genistein (Geni) for 30 min, 1 μM herbimycin A (Herb) for 2 h, 1 μM PP2 for 20 min, or transduced with adenoviruses expressing a dominant negative Ras (Neg-Ras) for 12 h (17). Cells transduced with the same amount of β-galactosidase (β-Gal) viruses were used as viral control. Both treated and control myocytes were then exposed to 100 μM ouabain. MAPK was measured after 5 min of ouabain exposure. Values are means ± SE of 4 different experiments. $^*P < 0.01$ vs. control.

Because binding and inhibition of $Na^+$-$K^+$-ATPase is the first step in ouabain regulation of $[Ca^{2+}]_i$, one could ask whether PD-98059 regulates $[Ca^{2+}]_i$ by affecting either the enzyme activity or its ouabain sensitivity. We addressed this issue by measuring the effects of PD-98059 as well as the other protein kinase inhibitors used here on both enzyme activity and its ouabain inhibition curve. ATPase assay was done using the purified canine kidney enzyme (37). The measurements showed no effect of these chemicals on either enzyme activity or the ouabain sensitivity (data not shown). The effects of the kinase inhibitors were also tested on the transport function of $Na^+$-$K^+$-ATPase, assayed as $^{86}Rb^+$ uptake in cultured cardiac myocytes (24, 36). We did not observe any significant effect of these inhibitors on ouabain-sensitive $^{86}Rb^+$ uptake (data not shown). Clearly, these chemicals affect downstream signaling pathways that are essential for ouabain to regulate $[Ca^{2+}]_i$ in cardiac myocytes.

To further test the role of p42/44 MAPKs in the ouabain regulation of $[Ca^{2+}]_i$, myocytes were transduced with an adenovirus expressing dominant negative $Ala^{221}$ MEK1 (MEK 1A). As shown in Fig. 9*A*,



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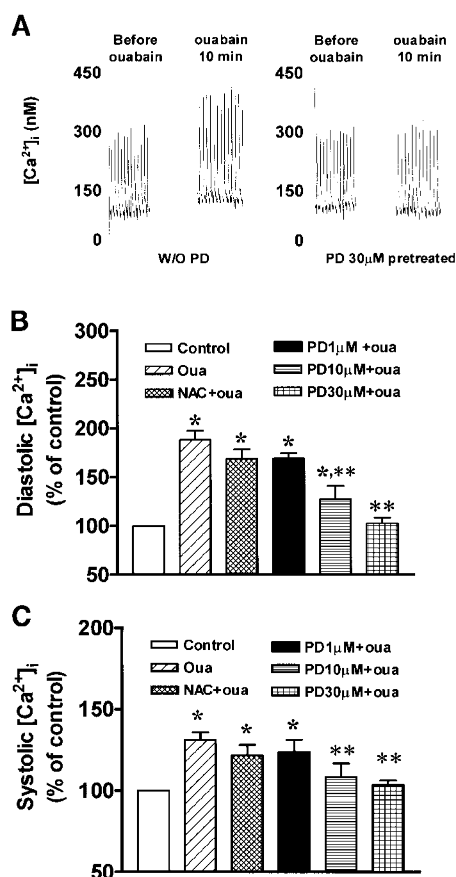


Fig. 8. Effects of PD-98059 and NAC on ouabain-induced increases in diastolic and systolic $[Ca^{2+}]_i$. Myocytes were pretreated with 10 mM NAC or different concentrations of PD-98059 for 30 min, exposed to 100 μM ouabain for 10 min, and then assayed for both diastolic and systolic $[Ca^{2+}]_i$ as in Fig. 1. *A*: representative trace showing that 30 μM PD-98059 blocks ouabain-induced increases in both diastolic and systolic $[Ca^{2+}]_i$. *B* and *C*: quantitative effects of PD-98059 and NAC on 100 μM ouabain-induced changes in diastolic (*B*) and systolic $[Ca^{2+}]_i$ (*C*). Values are means ± SE of 10–15 measurements from 4 different preparations. $^*P < 0.01$ vs. control; $^{**}P < 0.05$ vs. ouabain-treated control cells.

expression of MEK1A, like expression of $Asn^{17}$ Ras, blocked the ouabain-induced activation of p42 MAPK. When $[Ca^{2+}]_i$ was measured in response to 100 μM ouabain, expression of MEK1A also diminished the ouabain-induced increase in $[Ca^{2+}]_i$ in adult cardiac myocytes (Fig. 9*B*).

## DISCUSSION

Recently, we (10, 16, 21, 33) demonstrated that $Na^+$-$K^+$-ATPase of neonatal rat cardiac myocytes also functions as a signal transducer. It relays the message of extracellular ouabain to the various cellular compartments through several interrelated pathways, including activation of Src, EGFR, Ras, and p42/44 MAPKs, and increases in intracellular ROS production. Significantly, the effects of ouabain on protein tyrosine kinases and mitochondrial production of ROS are independent of changes in intracellular ion concentrations and contractility of these myocytes, which indicates that the enzyme exerts its signal-transducing function through its interaction with the neighboring membrane proteins (21). The findings presented here show that $Na^+$-$K^+$-ATPase is also a signal transducer in adult rat cardiac myocytes. Whereas prior studies (13, 24, 35) showed that increases in $[Ca^{2+}]_i$ due to inhibition of the ion-pumping function of the enzyme cooperate with increased ROS in regulation of cardiac genes, the present findings indicated that the signal-transducing function of the enzyme also contributes to ouabain-induced increases in $[Ca^{2+}]_i$ in cardiac myocytes. The new findings reveal the significance of $Na^+$-$K^+$-ATPase as a signal transducer in regulation of $[Ca^{2+}]_i$ in cardiac myocytes. These conclusions are summarized in Fig. 10 and discussed in details in the paragraphs that follow.

*Relationship between the signal-transducing function of $Na^+$-$K^+$-ATPase and the effects of ouabain on $[Ca^{2+}]_i$ in adult rat cardiac myocytes.* Cardiac myocytes from different species have different sensitivity to ouabain because these cells express different $Na^+$-$K^+$-ATPase isoforms (20). Whereas the human $\alpha_1$-isoform of $Na^+$-$K^+$-ATPase is highly ouabain sensitive, the rodent $\alpha_1$-isoform is ~1,000-fold less sensitive to ouabain. In rat cardiac myocytes, we showed that 10–100 μM ouabain caused ~20–50% inhibition of $Na^+$-$K^+$-ATPase in a dose-dependent manner (24, 36, 37). Under our experimental conditions, ouabain at concentrations up to 100 μM did not cause arrhythmic contraction and $Ca^{2+}$ overload in 15 min. It also had no effect on cell viability. On the other hand, these non-

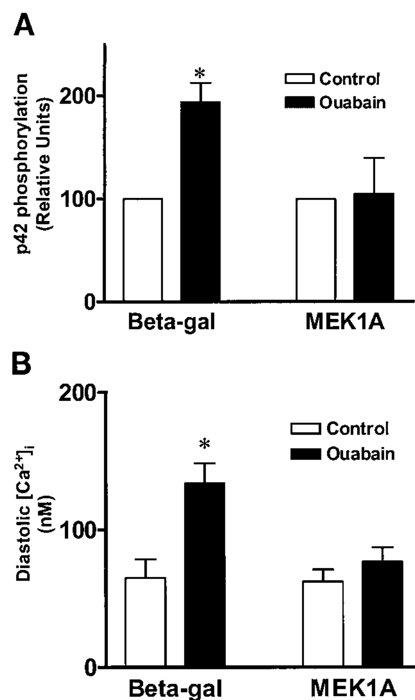


Fig. 9. Expression of a dominant negative MAPK kinase 1 (MEK1) ablates the effects of ouabain on both p42 MAPK and $[Ca^{2+}]_i$. Myocytes were transfected with either β-Gal or MEK1A viruses for 12 h and then exposed to 100 μM ouabain. Both p42 MAPK and $[Ca^{2+}]_i$ were determined as in Figs. 1 and 4. Values are means ± SE of 4 different preparations. $^*P < 0.01$ vs. control.



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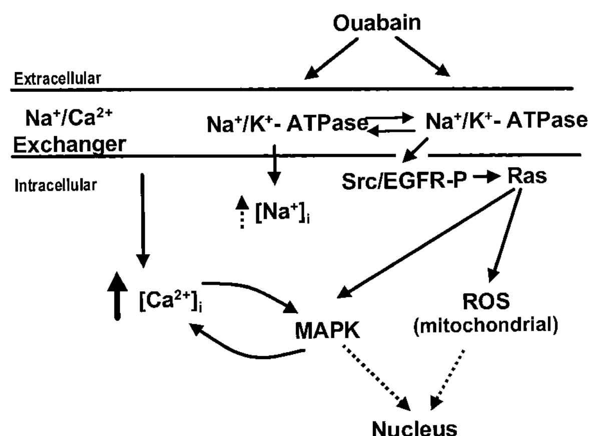


Fig. 10. Schematic presentation showing the pathways that connect the binding of ouabain to $Na^+$-$K^+$-ATPase and increases in $[Ca^{2+}]_i$. $[Na^+]_i$, intracellular $Na^+$ concentration; EGFR-P, phosphorylated epidermal growth factor receptor.

toxic concentrations of ouabain (10–100 μM) caused a rapid stimulation of protein tyrosine phosphorylation and activation of p42/44 MAPKs in adult rat cardiac myocytes (Figs. 3 and 4). It also raised intracellular ROS concentration in these cells (Fig. 5). It is important to note that the above signal-transducing function of the enzyme is not limited to cardiac myocytes but also found in other cells, including HeLa cells and A7r5 cells, which are derived from rat smooth muscles (10). Because the dose-response curves of ouabain effects on signal transduction (e.g., Fig. 4) correlate well with the ouabain inhibition curve on $Na^+$-$K^+$-ATPase in rat cardiac myocytes (24, 36, 37), it is likely that the signaling effects of ouabain are due to the interaction of ouabain with $Na^+$-$K^+$-ATPase. This notion is further supported by the following observations: when the dose-response curve of ouabain was determined in different cells, we found that the concentration curves of ouabain effects on signal transduction pathways correlated well with the ouabain sensitivities of different isoforms of $Na^+$-$K^+$-ATPase. For example, whereas 10 μM ouabain was required to stimulate tyrosine kinases and increase ROS production in rat cardiac myocytes, 10 nM ouabain was sufficient to cause similar effects in HeLa cells, which express highly ouabain-sensitive $Na^+$-$K^+$-ATPase (10, 21).

In both neonatal cardiac myocytes and A7r5 cells, we (10) demonstrated that ouabain binding to $Na^+$-$K^+$-ATPase activates Src kinase, which transactivates EGFR, resulting in activation of the Ras/Raf/MEK/MAPK cascade. The findings shown in Figs. 6 and 9 reveal that ouabain uses the same pathway in regulation of p42/44 MAPKs in adult rat cardiac myocytes. When $[Ca^{2+}]_i$ was measured in paced adult cardiac myocytes, ouabain raised both systolic and diastolic $[Ca^{2+}]_i$ (Figs. 1 and 2) in a time- and dose-dependent manner, which is consistent with earlier observations made in several different cardiac preparations (5, 11, 31). When the relation between the signal-transducing function of the enzyme and the effects of ouabain on $[Ca^{2+}]_i$ were determined (Figs. 6–9), we showed that inhibition of protein tyrosine kinases with either genistein or herbimycin A diminished ouabain-induced increases in $[Ca^{2+}]_i$. The fact that herbimycin A was effective in repressing the effects of ouabain on adult cardiac myocytes supports our proposition that Src family kinases play a pivotal role in initiating the signal-transducing function of $Na^+$-$K^+$-ATPase (10). This was further supported by the fact that PP2, a Src kinase-specific inhibitor, abolished the effects of ouabain on both p42/44 MAPKs and $[Ca^{2+}]_i$. Because Ras relays the signal from ouabain-induced activation of protein tyrosine kinases to several downstream pathways, including the activation of p42/44 MAPKs and the stimulation of mitochondrial production of ROS in cardiac myocytes, we reasoned that an activation of Ras may be essential for the effects of ouabain on $[Ca^{2+}]_i$ in adult cardiac myocytes. This notion was supported by the experiments shown in Fig. 7, in which the expression of dominant negative $Asn^{17}$ Ras abolished the ouabain-induced rise in $[Ca^{2+}]_i$ in adult cardiac myocytes. In cells other than cardiac myocytes, activation of p42/44 MAPKs is required for various stimuli-induced increases in $Ca^{2+}$ influx (7, 9, 23, 32). Increases in ROS stress are also capable of raising $[Ca^{2+}]_i$ in cardiac myocytes as well as in other types of cells (30). The results of the experiments shown in Fig. 8 indicate that activation of p42/44 MAPKs, but not stimulation of ROS production, relays activation of Ras to ouabain-induced increases in $[Ca^{2+}]_i$. This conclusion was further reaffirmed by the experiments shown in Fig. 9, showing that expression of dominant negative MEK1 ablated the effects of ouabain on $[Ca^{2+}]_i$. It is important to note that, although ROS are not involved in the effects of ouabain on $[Ca^{2+}]_i$, they play an essential role in ouabain-mediated regulations of cardiac genes and cell growth (35). Clearly, these new findings establish the necessity of the signal-transducing function of $Na^+$-$K^+$-ATPase for the effects of ouabain on $[Ca^{2+}]_i$ in adult rat cardiac myocytes.

*How does activation of p42/44 MAPKs contribute to ouabain-induced increases in $[Ca^{2+}]_i$?* Because p42/44 MAPKs regulate multiple effectors in cardiac myocytes, the simple answer is that the steps that link p42/44 MAPK to ouabain-induced increases in $[Ca^{2+}]_i$ remain to be established. However, based on the prior studies, it is appropriate to consider the following alternatives: because p42/44 MAPKs are involved in regulation of gene transcription, one could ask if activation of these kinases could affect protein levels of membrane transporters such as $Na^+$-$K^+$-ATPase. Indeed, our prior work (12, 35) showed that these kinases were involved in transcriptional regulation of the $\alpha_3$-subunit of $Na^+$-$K^+$-ATPase in cardiac myocytes. However, it took at least 12 h to see changes in $\alpha_3$ protein in these cells. Therefore, it is unlikely that changes in gene expression are involved in the ouabain-induced rapid increases in $[Ca^{2+}]_i$. During the early 1980s, several laboratories (17, 22) reported that cardiac glycosides at both therapeutic and toxic doses activated $Ca^{2+}$ channels in various cardiac preparations. Most significantly, it was speculated that activation of $Ca^{2+}$



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channels by ouabain involved a signal amplification process via protein kinases (22). Recently, activation of L-type $Ca^{2+}$ channels by ouabain has also been reported in cardiac myocytes as well as other cells (19, 38). Interestingly, a role of p42/44 MAPKs for regulation of the L-type $Ca^{2+}$ channel activity has been established recently (7, 8, 23). For example, in neuronal cells, MAPKs were found to be involved in phosphorylation of the $\alpha_1$-subunit of the channel (7, 8). In neonatal cardiac myocytes, the stimulation of L-type $Ca^{2+}$ channels by leukemia inhibitory factor was also related to the activation of p42/44 MAPKs (23). Because activation of MAPKs is required for the effects of ouabain on $[Ca^{2+}]_i$ (Figs. 8 and 9), we suggest that $Ca^{2+}$ channels may be activated by p42/44 MAPKs in response to ouabain, thus amplifying the effects of ouabain on $[Ca^{2+}]_i$. Alternatively, p42/44 MAPKs may alter the properties of $Na^+/Ca^{2+}$ exchanger so that a small change in intracellular $Na^+$ concentration can bring about a large change in $[Ca^{2+}]_i$. Clearly, these issues remain to be resolved in future studies. Nevertheless, as shown in Fig. 10, binding of ouabain to $Na^+$-$K^+$-ATPase not only inhibits the ion-pumping function of the enzyme, it also activates the signal-transducing function of the enzyme in adult cardiac myocytes. We believe that both functions of the enzyme are required for the ouabain regulation of $[Ca^{2+}]_i$ in cardiac myocytes. Although inhibition of the enzyme by ouabain can cause some increase in $[Ca^{2+}]_i$ by inhibition of $Na^+/Ca^{2+}$ exchanger-mediated $Ca^{2+}$ extrusion due to a small rise in intracellular $Na^+$(18, 26), activation of p42/44 MAPKs by ouabain will amplify the ouabain effects on $[Ca^{2+}]_i$ by altering the function of membrane transporters or ion channels. It is important to note that there is also cross-talk between $[Ca^{2+}]_i$ and p42/44 MAPKs, because increases in $[Ca^{2+}]_i$ can further activate p42/44 MAPKs (4, 16, 25). Therefore, increases in $[Ca^{2+}]_i$ and activation of p42/44 MAPKs may form a signal amplification loop in cardiac myocytes (Fig. 10), which supports the earlier speculation that the effects of ouabain on $[Ca^{2+}]_i$ are amplified by activation of protein kinases (22). Clearly, the mechanism of ouabain action on $[Ca^{2+}]_i$ involves a complex signaling network, including cross-talk among $Na^+$-$K^+$-ATPase, $Na^+/Ca^{2+}$ exchangers, possibly other membrane proteins as well as p42/44 MAPKs, and $[Ca^{2+}]_i$ in adult rat cardiac myocytes (Fig. 10).

In short, we demonstrated here that $Na^+$-$K^+$-ATPase functions as a signal transducer as well as an ion pump and that the signal-transducing function of the enzyme is essential for the effects of ouabain on $[Ca^{2+}]_i$ in rat cardiac myocytes. These findings underscore the biological significance of the signal-transducing function of the enzyme and warrant further studies on the nature of the enzyme as a signal transducer.

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