Signal-transducing function of Na\(^{+}\)-K\(^{+}\)-ATPase is essential for ouabain’s effect on \([\text{Ca}^{2+}]_i\) in rat cardiac myocytes

Jiang Tian, Xiaohua Gong, and Zijian Xie. Signal transducing function of Na\(^{+}\)-K\(^{+}\)-ATPase is essential for ouabain’s effect on \([\text{Ca}^{2+}]_i\), 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 \(\text{Ca}^{2+}\) concentration. 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 \(\text{Ca}^{2+}\) concentration. To prevent the ouabain-induced rise in \([\text{Ca}^{2+}]_i\), clearly, the activation of the Ras/MAPK cascade, but not ROS generation, is necessary for ouabain-induced increases in \([\text{Ca}^{2+}]_i\), in rat cardiac myocytes.

Na\(^{+}\)-K\(^{+}\)-ATPase is an energy-transducing ion pump in most mammalian cells. 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. This non-toxic concentration, 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 through the Na\(^{+}\)/Ca\(^{2+}\) exchanger.

Recently, studies 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 \([\text{Ca}^{2+}]_i\), also stimulate the nonproliferative growth (hypertrophy) of myocytes and regulate the transcription of a number of growth-related genes. 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. 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. Both pathways play an important role in ouabain regulation of cell growth and gene expression in neonatal cardiac myocytes.

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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 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\(^{2+}\)].

**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 as a probe. As previously reported (5a), we found that nontoxic concentrations of ouabain (up to 100 \(\mu\)M) caused no detectable changes in intracellular pH in these myocytes (data not shown).

**Preparation of replication-defective adenoviruses and adenovirus infection of cardiac myocytes.** Replication-defective adenoviruses expressing either a dominant negative Asn\(^{17}\) Ras or Ala\(^{221}\) MK1 virus were prepared and used for the infection of myocytes as described before (14, 16). An identical virus containing the \(\beta\)-galactosidase (\(\beta\)-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. 50% of myocytes were quiescent, and they were used for the experiments after an overnight culture.

**Fluorescence microscopic measurements of [Ca\(^{2+}\)].** Myocytes cultured on coverslips were perfused with the same medium containing 0.2 mg/ml collagenase and retrograde perfused for 15 min with Joklik medium. After 15 single myocytes from three independent preparations were imaged with an Attofluor imaging system (Atto Instruments) 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 \(\mu\)l 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 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin, and 50 mM Tris-HCl (pH 7.4). Cell lysates were centrifuged at 16,000 \(\times\) 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 \(\mu\)g/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\(^{221}\) MK1 virus were prepared and used for the infection of myocytes as described before (14, 16).

**Analysis of data.** Data are given as means \(\pm\) 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.
RESULTS

Time- and dose-dependent effects of ouabain on \([\text{Ca}^{2+}]_i\) in adult rat cardiac myocytes. Binding of ouabain to \(\text{Na}^+\text{-K}^+\)-ATPase inhibits the ion-pumping function of the enzyme. Our previous studies (24) measured the time-averaged changes in \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) in paced adult rat cardiac myocytes. As expected, ouabain raised both systolic and diastolic \([\text{Ca}^{2+}]_i\); 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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\); (Fig. 2) in these myocytes. This dose-response curve correlates well with the ouabain inhibition curve on rat cardiac \(\text{Na}^+\text{-K}^+\)-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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\). However, when cells were exposed to toxic concentrations of ouabain (e.g., > 200 μM), a large number of myocytes were \(\text{Ca}^{2+}\) 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.

\(\text{Na}^+\text{-K}^+\)-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 \(\text{Na}^+\text{-K}^+\)-ATPase and ouabain-induced increases in \([\text{Ca}^{2+}]_i\), 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. 4C) 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 \(\text{Na}^+\text{-K}^+\)-ATPase

![Fig. 1. Effects of ouabain on intracellular \(\text{Ca}^{2+}\) concentration \(([\text{Ca}^{2+}]_i)\) as a function of time in adult rat cardiac myocytes. To measure \(\text{Ca}^{2+}\) transients, cells were loaded with indo 1 and paced at 0.5 Hz. \([\text{Ca}^{2+}]_i\) was determined as described in MATERIALS AND METHODS. A: representative trace of \([\text{Ca}^{2+}]_i\) in a single cell. It was recorded after the cells were paced for 5 min and \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]_i\), as a function of time.

\({}^*P < 0.05\) and \({}^{**}P < 0.01\) vs. control.

![Fig. 2. Dose-response curve of the ouabain effect on \([\text{Ca}^{2+}]_i\). \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\), as in Fig. 1, in a dose-dependent manner. The effects of ouabain on diastolic \([\text{Ca}^{2+}]_i\), are presented, and values are means ± SE of 12 independent measurements from 4 experiments. \({}^*P < 0.05\) and \({}^{**}P < 0.01\) vs. control.

![Graph showing the dose-response curve of ouabain on intracellular calcium concentration.](http://www.ajpheart.org/ AJP-Heart Circ Physiol • VOL 281 • NOVEMBER 2001 • www.ajpheart.org)
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...
Ras is involved in the ouabain-induced increases in \([\text{Ca}^{2+}]_{i}\) under the same experimental conditions as in Fig. 6, in which adult cardiac myocytes were transduced with adenoviruses expressing a dominant negative Asn17 Ras. As shown in Fig. 7, expression of Asn17 Ras, but not \(\beta\)-Gal, abolished the effects of ouabain on \([\text{Ca}^{2+}]_{i}\). Clearly, the effects of ouabain on \([\text{Ca}^{2+}]_{i}\) 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 \([\text{Ca}^{2+}]_{i}\). 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 \([\text{Ca}^{2+}]_{i}\), 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 \(\mu\)M ouabain on both systolic and diastolic \([\text{Ca}^{2+}]_{i}\) in a dose-dependent manner. On the other hand, as shown in Fig. 8, \(\beta\) and \(\gamma\), although 10 mM NAC seemed to repress ouabain-induced increases in \([\text{Ca}^{2+}]_{i}\), 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 \([\text{Ca}^{2+}]_{i}\). Together, the above findings indicate that effects of ouabain on \([\text{Ca}^{2+}]_{i}\) in cardiac myocytes require the activation of the p42/44 MAPK pathway.

Because binding and inhibition of Na\(^+\)-K\(^+\)-ATPase is the first step in ouabain regulation of \([\text{Ca}^{2+}]_{i}\), one could ask whether PD-98059 regulates \([\text{Ca}^{2+}]_{i}\) 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 \(^{86}\text{Rb}^+\) uptake in cultured cardiac myocytes (24, 36). We did not observe any significant effect of these inhibitors on ouabain-sensitive \(^{86}\text{Rb}^+\) uptake (data not shown). Clearly, these chemicals affect downstream signaling pathways that are essential for ouabain to regulate \([\text{Ca}^{2+}]_{i}\) in cardiac myocytes.

To further test the role of p42/44 MAPKs in the ouabain regulation of \([\text{Ca}^{2+}]_{i}\), myocytes were transduced with an adenovirus expressing dominant negative Ala221 MEK1 (MEK 1A). As shown in Fig. 9A,
expression of MEK1A, like expression of Asn17 Ras, blocked the ouabain-induced activation of p42 MAPK. When \([\text{Ca}^{2+}]_i\) was measured in response to 100 \(\mu\text{M}\) ouabain, expression of MEK1A also diminished the ouabain-induced increase in \([\text{Ca}^{2+}]_i\) in adult cardiac myocytes (Fig. 9B).

**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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) in cardiac myocytes. The new findings reveal the significance of Na\(^+-\)K\(^+\)-ATPase as a signal transducer in regulation of \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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 a1-isoform of Na\(^+-\)K\(^+\)-ATPase is highly ouabain sensitive, the rodent a1-isoform is 1,000-fold less sensitive to ouabain. In rat cardiac myocytes, we showed that 10–100 \(\mu\text{M}\) 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 \(\mu\text{M}\) did not cause arrhythmic contraction and \([\text{Ca}^{2+}]_i\) overload in 15 min. It also had no effect on cell viability. On the other hand, these non-
inhibition of protein tyrosine kinases with either genistein or herbimycin A diminished ouabain-induced increases in \([\text{Ca}^{2+}]_i\). 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 \(\text{Na}^+\text{-K}^+\text{-ATPase}\) (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 \([\text{Ca}^{2+}]_i\). 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 \([\text{Ca}^{2+}]_i\) in adult cardiac myocytes. This notion was supported by the experiments shown in Fig. 7, in which the expression of dominant negative \(\alpha\text{S}\) Ras abolished the ouabain-induced rise in \([\text{Ca}^{2+}]_i\) in adult cardiac myocytes. In cells other than cardiac myocytes, activation of p42/44 MAPKs is required for various stimuli-induced increases in \([\text{Ca}^{2+}]_i\). Increases in ROS stress are also capable of raising \([\text{Ca}^{2+}]_i\); 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 \([\text{Ca}^{2+}]_i\). 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 \([\text{Ca}^{2+}]_i\). It is important to note that, although ROS are not involved in the effects of ouabain on \([\text{Ca}^{2+}]_i\), 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 \(\text{Na}^+\text{-K}^+\text{-ATPase}\) for the effects of ouabain on \([\text{Ca}^{2+}]_i\) in adult rat cardiac myocytes.

How does activation of p42/44 MAPKs contribute to ouabain-induced increases in \([\text{Ca}^{2+}]_i\)? 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 \([\text{Ca}^{2+}]_i\) 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 \(\text{Na}^+\text{-K}^+\text{-ATPase}\). Indeed, our prior work (12, 35) showed that these kinases were involved in transcriptional regulation of the \(\alpha\text{S}\) subunit of \(\text{Na}^+\text{-K}^+\text{-ATPase}\) in cardiac myocytes. However, it took at least 12 h to see changes in \(\alpha\text{S}\) protein in these cells. Therefore, it is unlikely that changes in gene expression are involved in the ouabain-induced rapid increases in \([\text{Ca}^{2+}]_i\). During the early 1980s, several laboratories (17, 22) reported that cardiac glycosides at both therapeutic and toxic doses activated \(\text{Ca}^{2+}\) channels in various cardiac preparations. Most significantly, it was speculated that activation of \(\text{Ca}^{2+}\) channels by cardiac glycosides is mediated through p42/44 MAPKs.

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