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Ouabain Interaction with Cardiac Na^+/K^+-ATPase Initiates Signal Cascades Independent of Changes in Intracellular Na^+ and Ca^{2+} Concentrations*

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Jiang Liu‡, Jiang Tian‡, Michael Haas‡, Joseph I. Shapiro§, Amir Askari‡, and Zijian Xie‡¶

From the Departments of ‡Pharmacology and §Medicine, Medical College of Ohio, Toledo, Ohio 43614

We have shown previously that partial inhibition of the cardiac myocyte Na^+/K^+-ATPase activates signal pathways that regulate myocyte growth and growth-related genes and that increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and reactive oxygen species (ROS) are two essential second messengers within these pathways. The aim of this work was to explore the relation between $[Ca^{2+}]_i$ and ROS. When myocytes were in a Ca^{2+}-free medium, ouabain caused no change in $[Ca^{2+}]_i$, but it increased ROS as it did when the cells were in a Ca^{2+}-containing medium. Ouabain-induced increase in ROS also occurred under conditions where there was little or no change in $[Na^+]_i$. Exposure of myocytes in Ca^{2+}-free medium to monensin did not increase ROS. Increase in protein tyrosine phosphorylation, an early event induced by ouabain, was also independent of changes in $[Ca^{2+}]_i$ and $[Na^+]_i$. Ouabain-induced generation of ROS in myocytes was antagonized by genistein, a dominant negative Ras, and myxothiazol/diphenyleneiodonium, indicating a mitochondrial origin for the Ras-dependent ROS generation. These findings, along with our previous data, indicate that increases in $[Ca^{2+}]_i$ and ROS in cardiac myocytes are induced by two parallel pathways initiated at the plasma membrane: One being the ouabain-altered transient interactions of a fraction of the Na^+/K^+-ATPase with neighboring proteins (Src, growth factor receptors, adaptor proteins, and Ras) leading to ROS generation, and the other, inhibition of the transport function of another fraction of the Na^+/K^+-ATPase leading to rise in $[Ca^{2+}]_i$. Evidently, the gene regulatory effects of ouabain in cardiac myocytes require the downstream collaborations of ROS and $[Ca^{2+}]_i$.

Ouabain and related cardiac glycosides are specific inhibitors of Na^+/K^+-ATPase, the enzyme that carries out the active transport of Na^+ and K^+ across the plasma membrane of most animal cells (1, 2). In the heart, interaction of cardiac glycoside drugs with the Na^+/K^+-ATPase regulates contractility. It is now widely accepted that partial inhibition of the cardiac myocyte Na^+/K^+-ATPase by a cardiac glycoside causes a modest increase in $[Na^+]_i$,[1] which in turn affects the plasma membrane Na^+/Ca^{2+}-exchanger, leading to a significant increase in $[Ca^{2+}]_i$ and in the force of contraction (3–5). This positive inotropic effect of cardiac glycosides is the basis of the major therapeutic use of these drugs in the management of congestive heart failure (3–6).

The cumulative findings of our laboratories during the past few years (7–12) have revealed the following previously unknown effects of ouabain on cardiac myocytes: 1) The same nontoxic concentrations of ouabain that cause partial inhibition of Na^+/K^+-ATPase and increase in $[Ca^{2+}]_i$ also stimulate the nonproliferative growth (hypertrophy) of the myocytes and regulate the transcription of a number of growth-related genes. 2) These ouabain effects involve the activation of multiple signal transduction pathways, including the activation of Src kinase and tyrosine phosphorylation of the epidermal growth factor receptor and other proteins, followed by the activation of Ras and the Ras/Raf/MEK/MAPK cascade. 3) The gene regulatory actions of ouabain, like its classical effect on cardiac contractility, are dependent on the net influx of Ca^{2+} and rise in $[Ca^{2+}]_i$, indicating that the latter is a shared second messenger for the ouabain effects on cardiac contractility and growth. 4) Ouabain's hypertrophic and gene regulatory effects also involve intracellularly generated ROS as essential second messengers. Significantly, our studies also showed (11) that antioxidants block the ouabain-induced ROS generation but not the ouabain-induced inhibition of the Na^+/K^+-ATPase and increase in $[Ca^{2+}]_i$, raising important questions regarding the relationship between the two essential second messengers of ouabain interaction with the cardiac Na^+/K^+-ATPase. For the clarification of the mechanism of the signal transducing function of the Na^+/K^+-ATPase, it seemed necessary to determine if the two second messengers, $[Ca^{2+}]_i$ and ROS, are generated sequentially or in parallel, and the order of their generations. The primary aim of the studies reported here was the resolution of these and related issues. A preliminary account of portions of this work has been presented (13).

EXPERIMENTAL PROCEDURES

Cell Preparation and Culture—The same protocol was used to prepare and culture neonatal ventricular myocytes as described in our previous work (7). In short, myocytes from 1-day-old Harlan Sprague-Dawley rats were isolated and purified on Percoll gradients. In a me-

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¶ To whom correspondence should be addressed: Dept. of Pharmacology, Medical College of Ohio, 3035 Arlington Ave., Toledo, OH 43614-5804. Tel.: 419-383-4182; Fax: 419-383-2871; E-mail: mheck@mco.edu.

[1] The abbreviations used are: $[Na^+]_i$, intracellular Na^+ concentration; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CM-DCF, 5(and 6)-chloromethyl-2′,7′-dichlorofluorescein; CM-DCFH, reduced CM-DCF; DMEM, Dulbecco's modified Eagle's medium; DPI, diphenyleneiodonium; $[K^+]_i$, intracellular K^+ concentration; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ROS, reactive oxygen species; SBFI, sodium binding benzofuran isophthalate; SBFI-AM, SBFI acetoxymethyl ester.

dium containing four parts of DMEM and one part Medium 199 (Life Technologies, Inc.), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum, myocytes were cultured for 24 h at 37 °C in humidified air with 5% CO_2. After 24 h, the myocytes were serum-starved for 48 h at which time all experiments were performed. Some of the experiments were done in a nominally Ca^{2+}-free medium, which was prepared to contain the same components as DMEM except that calcium salts were omitted and 0.1 mM EGTA was added to the medium. As we previously reported, incubation of cardiac myocytes in this medium had no significant effect on cell viability for the experimental durations used here (7). Immunofluorescence staining with a myosin heavy chain antibody showed that the cultures contained more than 95% myocytes.

Fluorescence Microscopic Measurements of $[Ca^{2+}]_i$, $[Na^+]_i$, and ROS—Myocytes were cultured on glass coverslips. $[Ca^{2+}]_i$ was measured by fura-2 as we previously described (7, 11). The fura-2 fluorescence was recorded using an Attofluor imaging system (Atto Instruments) at an excitation wavelength of 340/380 nm and at an emission wavelength of 505 nm. Under each experimental condition time-averaged signals were obtained from about 40 single cells. $[Ca^{2+}]_i$ was calculated based on the fluorescence ratio and the Ca^{2+}calibration curve (7, 11). To measure $[Ca^{2+}]_i$ transients, 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 International, Monmouth Junction, NJ). The probe was excited at 365 nm, and fluorescence emitted at 405 and 485 nm was recorded. The emission ratio was recorded at a speed of 60 Hz. $[Na^+]_i$ was determined by the fluorescent probe SBFI. Myocytes were loaded with 5 μM SBFI-AM and 0.075% Pluronic, a surfactant, for 30 min at 37 °C and washed. SBFI fluorescence was recorded as in the case of the fura-2 signal at an excitation wavelength of 340/380 nm and at an emission wavelength of 505 nm. Calibration of $[Na^+]_i$ was done by modifications of established procedures (14, 15). Briefly, SBFI-loaded myocytes were incubated in a solution containing 10 μM monensin, a Na^+-specific ionophore. Calibration solutions contained 0–80 mM Na^+. $[Na^+]_i$ in control and treated myocytes was then calculated based on this calibration curve. Substitution of 10 μM gramicidin for monensin caused no significant change in the calibration curve within the range of 0–80 mM Na^+. Intracellular ROS production in cells loaded with 10 μM CM-DCFH diacetate for 15 min at room temperature in the dark was measured as we previously described (11). Under each experimental condition, about 15 single myocytes were imaged with an Attofluor imaging system, and CM-DCF fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. All the chemicals for these fluorescence assays were obtained from Molecular Probes (Eugene, OR).

Measurement of Tyrosine Phosphorylation—Immunoblotting, using an anti-phosphotyrosine antibody with established specificity (PY99, Santa Cruz Biotechnology, Santa Cruz, CA), was performed as we described previously (12). The quantitative comparisons of the intensities of the bands were also done as described previously (12).

Preparation of Replication-defective Adenovirus Asn^{17} Ras and Adenovirus Infection of Cardiac Myocytes—A replication-defective adenovirus expressing the dominant negative Asn^{17} Ras was generated, amplified, purified, and used for the infection of myocytes as we described previously (10). An identical virus containing the β-galactosidase gene instead of the Asn^{17} Ras was used as the control (10).

RESULTS

Effects of Ouabain on ROS and $[Ca^{2+}]_i$ in Cardiac Myocytes—Our previous data (11) on the differential effects of an antioxidant on the ouabain-induced increases in $[Ca^{2+}]_i$ and ROS suggested that either the ouabain-induced ROS generation occurred within a pathway parallel to that causing the rise in $[Ca^{2+}]_i$, or that the increased $[Ca^{2+}]_i$ led to ROS generation, but that the antioxidant effect was distal to the rise in $[Ca^{2+}]_i$. To test these alternatives, ouabain effects on intracellular ROS generation were compared in myocytes incubated in the normal Ca^{2+}-containing culture medium and those incubated in a Ca^{2+}-free culture medium. The ouabain-induced ROS generation was nearly identical under the two conditions (Fig. 1). Because it is based on the accepted mechanism of ouabain action in cardiac myocytes (3–5), ouabain is not expected to increase $[Ca^{2+}]_i$ when myocytes are in a Ca^{2+}-free medium; therefore, the data in Fig. 1 suggest that ROS generation is independent from increased $[Ca^{2+}]_i$. To see if this could be

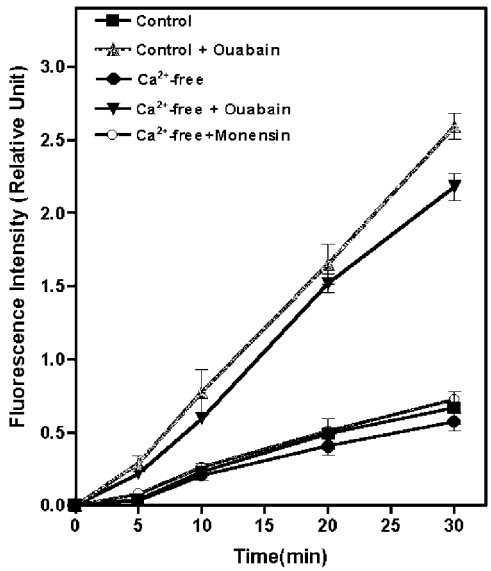

FIG. 1. **Effects of ouabain and monensin on intracellular ROS production by myocytes incubated in Ca^{2+}-containing (control) and Ca^{2+}-free media.** Myocytes loaded with CM-DCFH were incubated in the indicated media for 15 min, exposed to 100 μM ouabain or 25 μM monensin, and then measured for CM-DCF fluorescence as described under "Experimental Procedures." Each value is the mean ± S.E. of determinations on 40–60 cells in three to five independent experiments.

firmly established, we examined the effects of ouabain on $[Ca^{2+}]_i$ and on the spontaneous contractions of the myocytes exposed to the Ca^{2+}-containing and the Ca^{2+}-free media. As noted previously (7, 16, 17), the neonatal rat cardiac myocytes that are cultured at relatively low densities and serum-starved ("Experimental Procedures") are predominantly quiescent; *i.e.* most do not contract spontaneously, and some contract with a low rate. When these myocytes, in a normal Ca^{2+}-containing medium, were exposed to 100 μM ouabain, both the number of contracting cells and the rate of contractions increased. This is depicted in Fig. 2 where contractions in a population of cells, observed under a microscope, were counted before and after the addition of ouabain. The ouabain-induced increase in the rate of contraction was also observed when $[Ca^{2+}]_i$ transients, each representing a contraction, were monitored with indo-1 in single myocytes that were in the normal Ca^{2+}-containing medium, as shown in Fig. 3*A* for a representative single cell. Ouabain-induced increase in $[Ca^{2+}]_i$ was also evident in single cells (Fig. 3*A*), where diastolic $[Ca^{2+}]_i$ started to rise 2–3 min after exposure to ouabain. The time-averaged mean of $[Ca^{2+}]_i$ in cell populations, placed in the Ca^{2+}-containing medium and assayed after 20 min, nearly doubled in response to 100 μM ouabain as shown in Table I.

When myocytes were placed in the Ca^{2+}-free medium and monitored for beating, no contractions were noted either before or after the addition of ouabain (Fig. 2). This was also the case if the cells containing indo-1 were examined for $[Ca^{2+}]_i$ transients, as exemplified by the single cell tracing of Fig. 3*B*, where no increase in $[Ca^{2+}]_i$ and no transients were noted after ouabain addition. When $[Ca^{2+}]_i$ was measured in a population of myocytes placed in the Ca^{2+}-free medium, ouabain did not cause a change in $[Ca^{2+}]_i$ in sharp contrast to the ouabain-induced doubling of the $[Ca^{2+}]_i$ in myocytes placed in the Ca^{2+}-containing medium (Table I). The above data, taken together, clearly indicate that in the cultured cardiac myocytes used here ROS generation induced by 100 μM ouabain occurs independent of the ouabain-induced increase in $[Ca^{2+}]_i$ and the associated myocyte contraction.

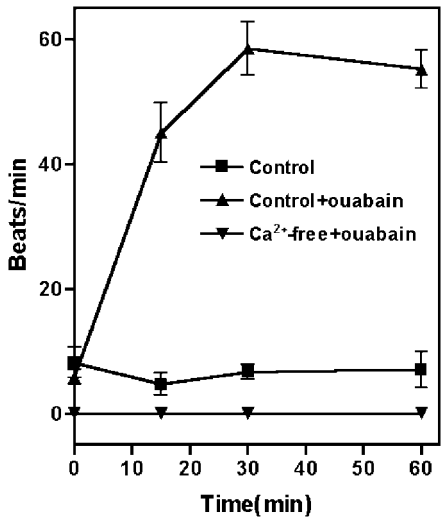

FIG. 2. **Effects of ouabain on myocyte contractile activity in Ca^{2+}-containing (control) and Ca^{2+}-free media.** Myocytes were incubated in the indicated media for 15 min then exposed to 100 μM ouabain for various times. Five myocytes per dish were randomly chosen, and spontaneous beating was counted under microscope for 30 s at each time point. A total of 15 cells from three dishes were counted in each experiment. Each value is the mean ± S.E. of determinations of 45 cells from three independent experiments. Myocytes exhibited no spontaneous beating when incubated in the Ca^{2+}-free DMEM (data not shown).

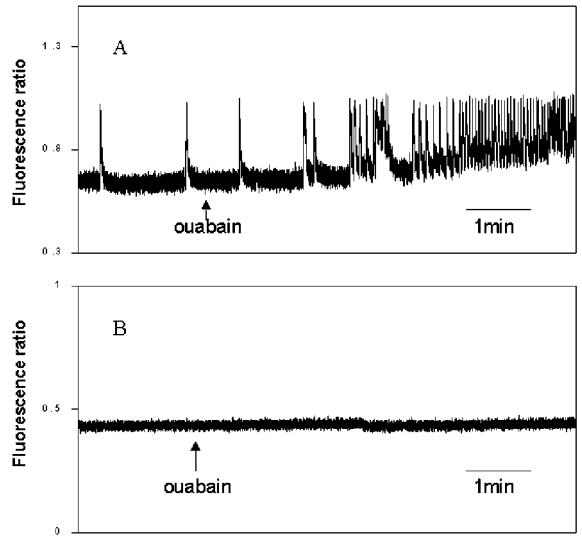

FIG. 3. **Effects of ouabain on $[Ca^{2+}]_i$ transients in myocytes.** Cells loaded with indo-1 were incubated in either the Ca^{2+}-containing (*A*) or the Ca^{2+}-free (*B*) medium for 15 min, exposed to 100 μM ouabain as indicated, and measured for transients as described under "Experimental Procedures." Each panel is a single cell tracing representative of those noted in four independent experiments.

Lack of Relation of the $[Na^+]_i$ to the Ouabain-induced ROS Generation in Myocytes—Based on the established reserve capacity of Na^+/K^+-ATPase in various intact heart preparations and in chick cardiac myocytes (4, 5, 18, 19), the partial inhibition of Na^+/K^+-ATPase caused by the highest ouabain concentration (100 μM) used in the present studies was expected to lead to either no increase or a modest increase in $[Na^+]_i$ of these myocytes (see "Discussion"). To see if this were indeed the case, we determined the effects of 100 μM ouabain on $[Na^+]_i$ when myocytes were incubated either in the Ca^{2+}-containing medium or in the Ca^{2+}-free medium (Fig. 4). As expected from previous studies on cardiac myocytes and isolated cardiac muscle preparations (20–22), exposure of the cells to the Ca^{2+}-free

TABLE I

Different effects of 100 μM ouabain on $[Ca^{2+}]_i$ in rat neonatal cardiac myocytes incubated in Ca^{2+}-containing and Ca^{2+}-free media

Cells were loaded with fura-2, placed either in the normal Ca^{2+}-containing medium or the Ca^{2+}-free medium for 15 min, exposed to ouabain, and monitored for changes in the fluorescence signals as indicated under "Experimental Procedures." The time-averaged mean of $[Ca^{2+}]_i$ in cell populations were determined 15–20 min after exposure to ouabain when $[Ca^{2+}]_i$ had stabilized.

Incubation medium	$[Ca^{2+}]_i$	
	Control	Ouabain-treated
Ca^{2+}-containing	109 ± 17	219 ± 28[a]
Ca^{2+}-free	86 ± 8	90 ± 12

[a] $p < 0.05$ relative to control.

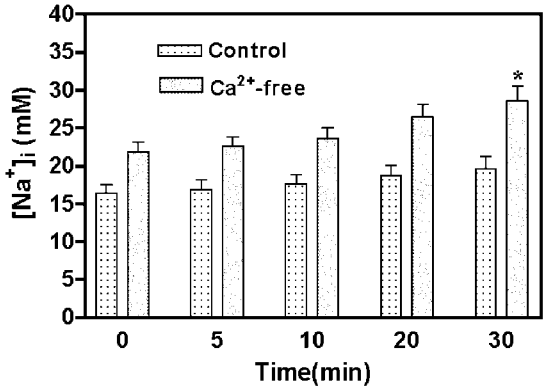

FIG. 4. **Time course of the effects of 100 μM ouabain on $[Na^+]_i$ in cardiac myocytes.** Cells loaded with BSFI were incubated in the Ca^{2+}-containing (control) or the Ca^{2+}-free medium for 15 min then exposed to ouabain for various times. $[Na^+]_i$ was measured as described under "Experimental Procedures." Each value is the mean ± S.E. of determinations from 30 different cells in three independent experiments. *$p < 0.05$, relative to zero time value in Ca^{2+}-free medium. At each time point the value for Ca^{2+}-free medium was significantly larger than the value in the corresponding control ($p < 0.05$).

medium caused a small increase in $[Na^+]_i$ prior to the addition of ouabain (Fig. 4). After the addition of ouabain, there were no significant changes in $[Na^+]_i$ during 30 min of incubation in the Ca^{2+}-containing medium, and only at one time point (30 min) a small but significant increase in $[Na^+]_i$ of the cells incubated in the Ca^{2+}-free medium was noted (Fig. 4). These findings are consistent with the degree of inhibition of Na^+/K^+-ATPase of these myocytes by 100 μM ouabain (see "Discussion"). Clearly, comparison of the data in Figs. 1 and 4 argues against the possibility of a ouabain-induced rise in $[Na^+]_i$ being responsible for the increased ROS generation. To see if a large increase in $[Na^+]_i$ affects ROS generation, cells incubated in the Ca^{2+}-free culture medium containing 150 mM Na^+ were exposed to monensin, a Na^+-specific ionophore. Monensin did not increase ROS generation (Fig. 1). This finding and the data of Fig. 4 rule out increased $[Na^+]_i$ as a second messenger for the ouabain-induced ROS generation in these myocytes when ouabain concentration is 100 μM or less.

Ouabain-induced Protein Tyrosine Phosphorylation in Myocytes Incubated in a Ca^{2+}-free Medium—We have shown that in neonatal cardiac myocytes ouabain, added to the normal Ca^{2+}-containing culture medium, causes a rapid increase in protein tyrosine phosphorylation (12). When myocytes were placed in the Ca^{2+}-free medium, ouabain also induced rapid increases in tyrosine phosphorylation of a number of proteins (Fig. 5). This result, and the data of Table I and Figs. 3*B* and 4, clearly indicate that stimulation of tyrosine phosphorylation by 100 μM ouabain is also not due to changes in $[Ca^{2+}]_i$ or $[Na^+]_i$. Although the prominent tyrosine-phosphorylated bands of Fig. 5 seem to be similar to those noted in experiments done in the

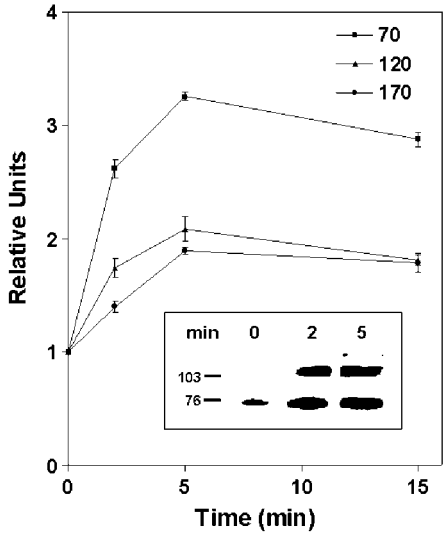

FIG. 5. **Time-dependent effects of 100 μM ouabain on tyrosine phosphorylation of several proteins of myocytes incubated in the Ca^{2+}-free medium.** Myocytes cultured in the normal medium were incubated in the Ca^{2+}-free medium for 15 min before being exposed to ouabain for the indicated durations. Cell lysates were prepared, and 60 μg of protein/lane was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with a monoclonal anti-phosphotyrosine antibody as described under "Experimental Procedures" and elsewhere (12). Quantitative comparisons of the intensities of three prominent bands, with the indicated apparent kDa values, were also done as previously described (12), using appropriate exposure times for the different bands. The indicated values are mean ± S.E. of four independent experiments. The *inset* is a representative blot of three prominent tyrosine-phosphorylated bands.

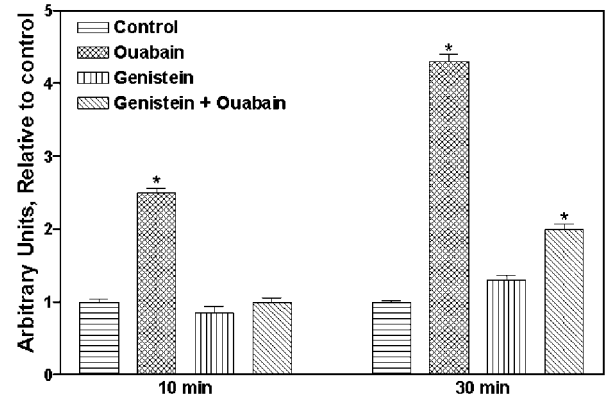

FIG. 6. **Effects of genistein on ouabain-induced ROS production.** Myocytes cultured in the normal Ca^{2+}-containing medium were preincubated with 100 μM genistein for 30 min, exposed to 100 μM ouabain for 10 or 30 min, and then assayed for ROS as in Fig. 1. Each value is the mean ± S.E. of determinations from 30–40 cells in three independent experiments. At each time point, values are expressed relative to the control value of one at that time. $*p < 0.05$.

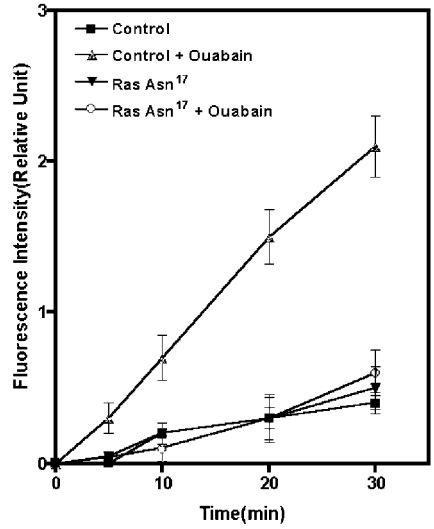

FIG. 7. **Effect of the expression of the dominant negative Ras on the ouabain-induced ROS production in cardiac myocytes.** Myocytes were transduced with either Ras Asn^{17} or a control virus for 12 h as indicated under "Experimental Procedures," then exposed to 100 μM ouabain and assayed for ROS as in Fig. 1. Each value is the mean ± S.E. of 40–50 determinations in four independent experiments.

Ca^{2+}-containing medium (Figs. 2–5 of Ref. 12) and because we have not done a thorough comparison of the phosphorylation patterns obtained in the Ca^{2+}-containing and Ca^{2+}-free media, the possibility of the existence of subtle but significant differences in these patterns cannot be ruled out and remains to be explored.

Relation of the Ouabain-induced ROS Generation in Myocytes to Protein Tyrosine Phosphorylation and Ras—Because the ouabain-induced increases in tyrosine phosphorylation and ROS generation were both found to be independent of increases in $[Ca^{2+}]_i$ and $[Na^+]_i$, we explored the relation of the two ouabain-induced events. Genistein blocked the ouabain-induced ROS production (Fig. 6), suggesting that increased protein tyrosine phosphorylation, which leads to Ras activation (12), is required for ROS production. This conclusion could be questioned, however, due to the possibility of genistein also being an antioxidant (23). Seeking independent evidence for the suggested conclusion of the experiments of Fig. 6, we tried to determine if the ouabain-induced ROS production required activated Ras. Using procedures that we have used previously (10), myocytes were transfected with an adenovirus expressing a dominant negative Ras or a control virus, exposed to ouabain, and assayed for intracellular ROS. As shown in Fig. 7, ouabain stimulated ROS production in control myocytes but not in those expressing the dominant negative Ras, thus indicating that ROS production is Ras-dependent.

The Sources of the Ouabain-induced ROS—To begin the identification of the intracellular origin of ROS generated in response to ouabain, myocytes were preincubated with different concentrations of myxothiazol (an inhibitor of ROS generated in mitochondria) or DPI (an inhibitor of flavoenzymes), then exposed to ouabain, and monitored for ROS production. In preliminary experiments (not shown) each compound was found to inhibit the ROS generation, and the maximally effective concentrations were found to be 0.6 μM myxothiazol and 10 μM DPI. At these optimal concentrations, each compound blocked a fraction of the ouabain-induced ROS generation, but the combination of the two caused complete inhibition of the ouabain effect (Fig. 8). These data, along with those of Fig. 7, indicate that ouabain-induced and Ras-dependent ROS originate, at least in part, from the myocyte mitochondria. Evidently, the ROS-generating pathways that begin at the plasma membrane Na^+/K^+-ATPase and are independent of changes in $[Ca^{2+}]_i$ and $[Na^+]_i$ extend into the mitochondrial matrix.

Ouabain-induced ROS Generation in Cells Other Than Cardiac Myocytes—Because our recent studies (12) showed that the ouabain-induced increase in protein tyrosine phosphorylation occurred in cardiac myocytes and several other cell types, it was of interest to know the cell type specificity of the ouabain-induced ROS generation. In experiments similar to those of Fig. 1, ouabain was found to stimulate ROS generation in A7r5 cells (not shown) and in HeLa cells (Fig. 9). It is of particular interest that the effective ouabain concentrations for ROS generation in HeLa cells are about two orders of magnitude lower than the effective concentrations in rat cardiac myocytes (Fig.

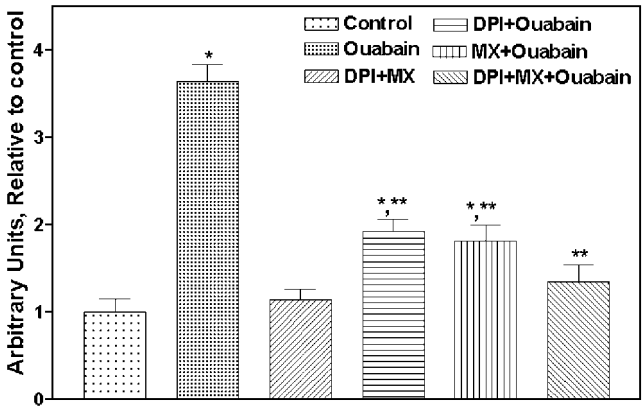

FIG. 8. **Effects of DPI and myxothiazol on the ouabain-induced ROS production in cardiac myocytes.** Myocytes were pretreated with either 20 μM DPI or 0.6 μM myxothiazol (*MX*) or the combination of the two for 15 min then exposed to 100 μM ouabain. Intracellular ROS were measured after 30 min of exposure to ouabain as in Fig. 1. Each value, expressed relative to the control value of one in the absence of ouabain, is the mean ± S.E. of 40–50 determinations in four independent experiments. *$p < 0.05$ in comparison with control; **$p < 0.05$ in comparison with ouabain-treated cells in the absence of DPI and MX.

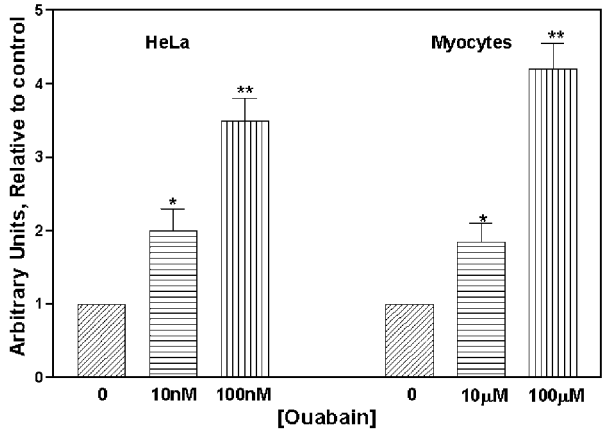

FIG. 9. **Dose-dependent effects of ouabain on the generation of ROS in rat neonatal cardiac myocytes and HeLa cells.** Cells were exposed to the indicated concentrations of ouabain, and intracellular ROS were measured as in Fig. 1 after 30 min of exposure. Each value, expressed relative to the control value of one in the absence of ouabain, is the mean ± S.E. of 40–50 determinations in four independent experiments. *$p < 0.05$; **$p < 0.01$.

9 and Ref. 11). This is in keeping with the established differences in the ouabain sensitivities of the predominant Na^+/K^+-ATPase isoforms of these cells (2, 12).

In view of the findings of Fig. 9, limited experiments were done to assess the possible relation of ouabain-induced ROS generation in HeLa cells to $[Ca^{2+}]_i$ and $[Na^+]_i$. As evident from the data of Fig. 10, after 20 min of exposure of the cells to 0.1 μM ouabain in the normal Ca^{2+}-containing culture medium, when a large increase in ROS was noted, there were no significant changes in $[Na^+]_i$ and $[Ca^{2+}]_i$. These findings reinforce the conclusions of the experiments on myocytes by showing that, with ouabain concentrations that cause partial inhibition of Na^+/K^+-ATPase, it is possible to induce ROS generation without significant increases in $[Ca^{2+}]_i$ or $[Na^+]_i$.

DISCUSSION

In our previous studies (7–12) we have shown that the plasma membrane Na^+/K^+-ATPase of the cardiac myocyte acts as a signal transducer by relaying the message of its interaction with extracellular ouabain to the cell nucleus through several interrelated pathways. We have also identified portions

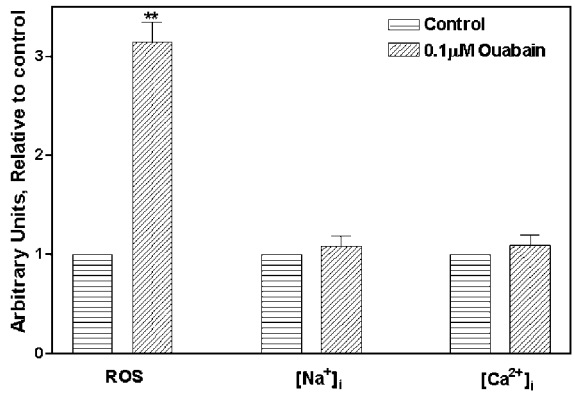

FIG. 10. **Effects of 0.1 μM ouabain on ROS generation, $[Na^+]_i$ and $[Ca^{2+}]_i$ in HeLa cells.** Cells cultured in the normal Ca^{2+}-containing medium were loaded with the appropriate fluorescent probes, exposed to ouabain for 20 min, and assayed as indicated under "Experimental Procedures." Each value in the ouabain-treated cell population is expressed relative to control value in cells not exposed to ouabain. **$p < 0.01$. The calculated values of ion concentrations were: $[Na^+]_i$, 12.6 ± 1.5 mM in the control and 13.7 ± 3.9 mM in the presence of ouabain; $[Ca^{2+}]_i$, 71.7 ± 5.7 nM in the control and 79 ± 9 nM in the presence of ouabain.

of these pathways, including segments that are close to ouabain's interaction with the Na^+/K^+-ATPase and involve Src, the epidermal growth factor receptor, and increased protein tyrosine phosphorylation. The findings presented here establish that such a proximal cascade that includes protein tyrosine phosphorylation followed by the increased generation of intracellular ROS, is independent of the increases in $[Ca^{2+}]_i$ and $[Na^+]_i$ that are the expected consequences of ouabain interaction with the cardiac Na^+/K^+-ATPase. The logical conclusion is that the Na^+/K^+-ATPase has two distinct roles within the plasma membrane: One being its classical function as an ion pump, and the other, as a signal transducer through protein-protein interactions. This and the other conclusions discussed below are summarized in Fig. 11.

Ouabain-induced Increases in ROS Generation and Protein Tyrosine Phosphorylation Are Independent of Increases in $[Ca^{2+}]_i$ and $[Na^+]_i$—When myocytes are placed in a Ca^{2+}-free medium, there is no ouabain-induced increase in $[Ca^{2+}]_i$ (Figs. 2 and 3, and Table I), but there are ouabain-induced increases in ROS generation and protein tyrosine phosphorylation (Figs. 1 and 5), which indicate that these events do not require the ouabain-induced increase in $[Ca^{2+}]_i$ that is noted when myocytes are in a Ca^{2+}-containing medium (Fig. 3 and Table I). The irrelevance of the increase in $[Na^+]_i$ to the above ouabain-induced signaling events is also established convincingly by the findings that, at the ouabain concentration used, with or without the presence of extracellular Ca^{2+}, changes in $[Na^+]_i$ are nonexistent or barely detectable (Fig. 4) and by the lack of effect of monensin on ROS production (Fig. 1). The data on $[Na^+]_i$ also suggest that a role for the altered $[K^+]_i$ is unlikely, because any ouabain-induced change in $[Na^+]_i$ is expected to be accompanied by a smaller ouabain-induced change in $[K^+]_i$. Taken together, the data of Table I and Figs. 1–5 show that, at the ouabain concentrations used, the induced pathways leading to increased protein tyrosine phosphorylation and ROS generation are parallel to those leading to a small increase in $[Na^+]_i$, which in turn leads to a more prominent rise in $[Ca^{2+}]_i$ through the altered function of the Na^+/Ca^{2+}-exchanger (3–5) as depicted in Fig. 11.

Two other important aspects of the data of Table I and Figs. 1–5 need further consideration: 1) The highest ouabain concentration (100 μM) we have used in the experiments on neonatal rat cardiac myocytes to activate signal cascades does not affect

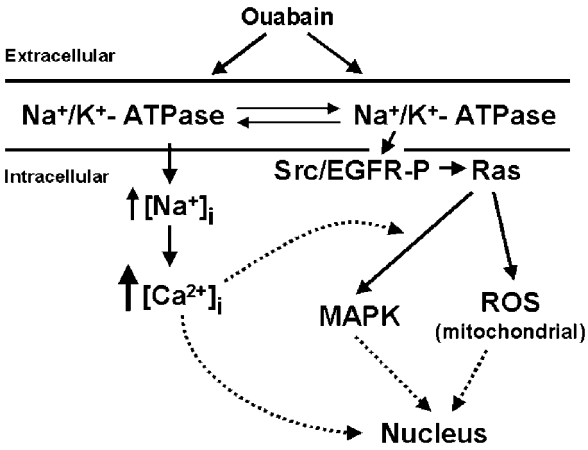

FIG. 11. **Schematic representation of the Ca^{2+}-independent and the Ca^{2+}-dependent pathways that are linked to the cardiac myocyte Na^+/K^+-ATPase and are activated in response to ouabain.** See "Discussion" and Ref. 12.

myocyte viability (7) and causes about 50% inhibition of the Na^+/K^+-ATPase activity and the associated transport function in these cells (7, 24). At first, it may seem odd that this degree of inhibition of the Na^+/K^+-ATPase leads to so little change in the steady-state $[Na^+]_i$ as shown in Fig. 4 and also noted previously in neonatal rat cardiac myocytes (15). This apparent discrepancy, which has been observed in various cardiac preparations, is due to the well established reserve capacity of the Na^+/K^+-ATPase with respect to the need for the maintenance of normal Na^+ and K^+ gradients (4, 5, 18, 19). A number of early studies have demonstrated that more than about 50% inhibition of cardiac Na^+/K^+-ATPase by a cardiac glycoside is required before significant changes in $[Na^+]_i$ begin to occur (4, 5, 18). Interestingly, recent studies not involving cardiac glycosides have also indicated the presence of an excess of Na^+/K^+-ATPase in the rat heart by showing that the depression of more than 40% of the enzyme content is needed before changes in normal Na^+ and K^+ gradients are noted (25). The existence of this excess capacity is also pertinent to the main conclusion of the present work regarding the dual functions of the Na^+/K^+-ATPase (Fig. 11), which suggests that the signal-transducing function of the enzyme through transient stimulus-induced protein-protein interactions need not interfere with the housekeeping function of the enzyme as an ion pump. 2) A number of studies on the regulation of cardiac hypertrophy have suggested that contraction *per se* may be a stimulus for the initiation of the various gene regulatory signaling pathways (Ref. 17 and references therein). Our experiments on myocytes in Ca^{2+}-free media (Figs. 1–5), showing ouabain-induced effects on protein tyrosine phosphorylation and ROS production in quiescent myocytes, also rule out contraction, or the altered force of contraction, as the primary inducer of the signal pathways, at least for those segments that lead to ROS generation.

Our limited studies on cells other than cardiac myocytes (Figs. 9 and 10, and Ref. 12) suggest that the entire pathway that links Na^+/K^+-ATPase to ROS generation (Fig. 11) is not limited to myocytes. Significantly, the findings on HeLa cells (Fig. 10) strongly support the conclusions of the myocyte studies by showing ouabain-induced ROS generation in the absence of increases in $[Ca^{2+}]_i$ and $[Na^+]_i$. The ouabain concentration used in these studies (0.1 μM) inhibits the hydrolytic and transport functions of Na^+/K^+-ATPase of the wild-type HeLa cells by about 40% (26). That $[Ca^{2+}]_i$ is not changed by ouabain (Fig. 10) is in keeping with the known absence of Na^+/Ca^{2+}-exchanger in HeLa cells (27). The absence of a significant effect of this concentration of ouabain on $[Na^+]_i$ (Fig. 10) suggests that HeLa cells, like myocytes, have an excess of Na^+/K^+-ATPase in regard to the need for the maintenance of normal Na^+ and K^+ gradients. We know of no previous findings that argue for or against the existence of such excess in HeLa cells.

The ROS-generating Pathway of Cardiac Myocytes Extends from the Na^+/K^+-ATPase to the Mitochondria through Ras—The blockade of ouabain-induced ROS production by genistein (Fig. 6), coupled with the data on dominant negative Ras (Fig. 7), suggests that the Ras-dependent ROS generation is distal to increases in protein tyrosine phosphorylation and the associated Src activation that are detailed in the accompanying paper (12). Because in the absence of extracellular Ca^{2+} the ouabain-induced ROS generation persists (Fig. 1), but not the ouabain-induced MAPK activation (10), we hypothesize two branches beginning at Ras: one being the Ras/Raf/MEK/MAPK cascade, and the other leading to ROS generation (Fig. 11). A possible mechanism for the regulation of the Ras/MAPK cascade by rise in $[Ca^{2+}]_i$ is an effect of a Ca^{2+}/calmodulin-dependent protein kinase on MEK, suggested in cells other than myocytes (28).

The partial inhibition of ouabain-induced ROS generation by either myxothiazol or DPI and the complete inhibition by the combination of the two (Fig. 8) are informative regarding the sources of ROS. Myxothiazol is an inhibitor of the mitochondrial site III electron transport (29, 30), but DPI may block ROS generation through the mitochondrial complex I or several other cellular sources, including NAD(P)H oxidase (31). Our data, therefore, implicate the mitochondria as a source of the ouabain-induced ROS, but do not rule out other cellular sources. Possible mechanisms for the extension of the ouabain-induced pathways to the mitochondria include the Ras/ceramide-mediated stimulation of the mitochondrial ROS generation, for which there is evidence in cells other than cardiac myocytes (32, 33). Regardless of the mechanistic details, the present findings permit the important conclusion that signal pathways initiated at Na^+/K^+-ATPase through protein-protein interactions extend to the mitochondria to generate ROS.

Relation between $[Ca^{2+}]_i$ and ROS as Second Messengers—In cardiac myocytes, and perhaps in all other cells that also express significant amounts of the plasma membrane Na^+/Ca^{2+}-exchanger, the most prominent early effect of the gradual inhibition of the Na^+/K^+-ATPase on intracellular ions is the rise in $[Ca^{2+}]_i$ rather than changes in $[Na^+]$i and $[K^+]_i$ (3–5, 18, 19). With the partial inhibition of the enzyme, this increased $[Ca^{2+}]_i$, the source of which is the extracellular Ca^{2+}, not only regulates contractility, but also the growth and the gene regulatory effects that are associated with the partial inhibition of Na^+/K^+-ATPase, as we have shown previously (7–10). The present demonstration, that large segments of the proximal signaling events emanating from Na^+/K^+-ATPase do not require the rise in $[Ca^{2+}]_i$, clearly indicates that this rise is not an early and all-important second messenger for the gene regulatory role of the cardiac Na^+/K^+-ATPase, but that it cooperates with the increased ROS to regulate distal events and cross-talk among the pathways (Fig. 11).

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