Positive Inotropic Effect of Ouabain on Isolated Heart Is Accompanied by Activation of Signal Pathways that Link Na^+/K^+-ATPase to $ERK_{1/2}$

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Abstract: Exposure of cultured rat cardiac myocytes to ouabain is known to cause the interaction of Na^+/K^+-ATPase with adjacent proteins, leading to activation of multiple signal transduction pathways, regulation of growth-related genes, and hypertrophy. The aim of this work was to determine if the proximal signaling events identified in cultured myocytes also occur in isolated intact hearts of rat and guinea pig in response to positive inotropic doses of ouabain. Langendorff rat heart preparations were exposed to 50 μ*M* ouabain to produce positive inotropy without toxicity, and assayed for Src kinase, protein kinase C, and extracellular signal-regulated kinases 1 and 2 ($ERK_{1/2}$). These kinases were rapidly activated by ouabain as in cultured cells. In isolated guinea pig hearts, 1 μ*M* ouabain caused $ERK_{1/2}$ activation comparable to the effect of 50 μ*M* ouabain in rat heart and consistent with the higher ouabain sensitivity of the contractility of guinea pig heart. These data show that the proximal ouabain-induced signal pathways previously noted in cultured cells are not artifacts of dispersion/culturing of myocytes, and are not the peculiar properties of the rat heart with its relatively low ouabain sensitivity. They also suggest that treatment with positive inotropic doses of cardiac glycosides is likely to be associated with changes in the cardiac phenotype.

Key Words: Cardiac glycosides–Extracellular signal-regulated kinase–Na^+/K^+-ATPase–Ouabain–Protein kinase C–Src kinase.

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Ouabain and related cardiac glycosides are highly specific inhibitors of the Na^+/K^+-ATPase (the sodium pump) that catalyzes the coupled active transport of Na^+ and K^+ across the plasma membrane of most animal cells (1). It is now generally accepted that the positive inotropic effect of a cardiac glycoside on the myocardium is due to the partial inhibition of the sarcolemmal Na^+/K^+-ATPase, causing a small increase in intracellular Na^+, which in turn affects the neighboring Na^+/Ca^{2+}-exchanger, leading to a significant increase in intracellular Ca^{2+} and in the force of contraction (2,3).

In recent years, we have shown that in primary cultures of cardiac myocytes prepared from the neonatal or the adult rat heart, ouabain interaction with Na^+/K^+-ATPase initiates protein-protein interactions that cause the activation of Src kinase, the transactivation of epidermal growth factor receptor, the stimulation of multiple signal pathways that lead to the transcriptional regulation of growth-related genes, and the hypertrophic growth of the myocytes (4–11). We have also established that these dose-dependent effects of ouabain on cultured myocytes may be obtained at drug concentrations that cause partial inhibition of the sodium pump and nontoxic increases in intracellular Ca^{2+} (4,8,10), clearly suggesting that ouabain's classic effect on cardiac contractility is accompanied by the activation of these signaling events.

In spite of the established value of the cultured cardiac myocytes as a model of the intact heart, there is always a legitimate concern that a phenomenon first observed in this model may be an artifact of the dispersion and culturing of myocytes, and therefore, may either not occur or not be faithfully duplicated in the intact heart. Specifically, there is evidence that activations of some signal transduction pathways by extracellular stimuli in cultured cardiac myocytes are indeed not duplicated in the intact heart (12–14). Based on these considerations, we deemed it necessary to determine if the rapid early sequences of the previously mentioned ouabain-induced signaling events that we had observed in cultured rat cardiac myocytes (10) are also noted in the isolated intact heart. The results of the initial studies toward this aim are reported here, using Langendorff preparations of rat and guinea pig hearts. Studies on the latter were included to address another serious concern; i.e., the possibility that the ouabain-activated signal

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transduction pathways observed in rat heart and cardiac myocytes may be due to the relatively low sensitivities of these preparations to cardiac glycosides.

METHODS

Isolated Heart Preparations

Male Sprague-Dawley rats (300–350 g) and Harlan guinea pigs (250–300 g) were killed in accordance with institutional policies. Conventional isolated Langendorff preparations were set up to measure left ventricular pressure and determinate the rate of left ventricular pressure increase ($+dp/dt_{max}$) as an index of contractility according to the same procedures described by Onwochei (15). The perfusion buffer used was the normal Krebs-Henseleit solution containing 118 m*M* NaCl, 4.7 m*M* KCl, 0.8 m*M* $MgSO_4$, 1.3 m*M* $CaCl_2$, 0.3 m*M* EGTA, 1.2 m*M* KH_2PO_4, 25 m*M* $NaHCO_3$, 11 m*M* glucose and equilibrated with 95% O_2 and 5% CO_2 (pH 7.4) at 37°C. Each heart was perfused for 30 min with the control buffer before contractility measurements were recorded. To determine ouabain effects, the control perfusion buffer was switched to the same buffer containing the indicated ouabain concentrations. Control and ouabain-treated Langendorff hearts were quick-frozen at indicated times in liquid nitrogen and powdered ventricular samples were used to prepare lysates for the following assays.

Assay of PKC Activation/Translocation

The frozen ventricular sample (100 mg wet weight) was suspended in 1 ml of an ice-cold solution containing 10 m*M* EGTA, 1 m*M* EDTA, 0.5 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride, 50 µg leupeptin, 25 µg aprotinin, and 20 m*M* Tris-HCl (pH 7.5). The suspension was homogenized in a Potter-Elvehjen homogenizer, and centrifuged at 100 000*g* for 1 h at 4°C. The supernatant, designated as the cytosolic fraction, was removed and saved. The pellet was homogenized in the solution to which 1% Triton X-100 was added, placed on ice for 30 min, and centrifuged at 25 000*g* for 10 min. The supernatant, designated as the particulate fraction, was saved.

The cytosolic and particulate fractions were passed through DE-52 cellulose columns (10), and assayed for PKC activity, using histone H_1 as substrate, in reaction mixtures containing 20 m*M* Tris-HCl (pH 7.5), 10 µ*M* [γ-^{32}P]ATP, 10 m*M* magnesium acetate, 0.75 m*M* $CaCl_2$, 50 µg/ml leupeptin, 100 µg/ml histone, with or without 30 µM phosphatidylserine, and 0.5 µ*M* dioleyl-sn-glycerol. The initial rate of the formation of the ^{32}P-labeled histone that was dependent on phosphatidylserine/diacylglycerol was determined as previously described (10).

Assay of Src Kinase Activity

The frozen ventricular sample (10–20 mg wet weight) was suspended in 1 ml of an ice-cold solution containing 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, 10 µg aprotinin, 10 µg leupeptin, 1 m*M* Na_3VO_3, 1 m*M* NaF, 1 m*M* okadaic acid, 1% NP40, 0.25% sodium deoxycholate, and 50 m*M* Tris-HCl (pH 7.4). The suspension was homogenized with 20 strokes of a tight-fitting Dounce homogenizer, rotated for 15 min at 4°C, and centrifuged at 100 000*g* for 10 min. The supernatant was mixed with 8 µg monoclonal anti-Src antibody, gently rotated overnight at 4°C, mixed with Protein G agarose beads, and rotated for 2 h at 4°C. The beads were then washed once with the buffer and twice with phosphate-buffered saline. One third of the beads was extracted by boiling in SDS-PAGE sample buffer, subjected to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the polyclonal anti-Src antibody, and the appropriate secondary antibody. Quantitation of the relative intensities of the immunoblots was done using chemiluminescence and densitometry as described previously (6,10). The remainder of the agarose beads containing the immunoprecipitated Src were used for the assay of Src kinase by a commercial kit, involving the measurement of the initial rate of transphosphorylation of a specific peptide substrate by [γ-^{32}P]ATP, as indicated previously (7). Src kinase activity of each sample was expressed as the ratio of this initial rate per unit of Src protein as determined in the Western blots, in relative units.

Assay of Phosphorylation/Activation of $ERK_{1/2}$

The frozen ventricular sample (100 mg wet weight) was suspended in 1 ml of an ice-cold solution containing 250 m*M* sucrose, 30 m*M* histidine, 1 m*M* EDTA, 1 m*M* phenylsulfonyl fluoride, 25 µg aprotinin, 50 µg leupeptin, 0.25 m*M* dithiothreitol, and 1% Triton X-100 (pH 6.8). The suspension was homogenized in a Dounce homogenizer, rotated for 90 min at 4°C, and centrifuged at 8000*g* for 5 min. Equal amounts of protein from the supernatant were subjected to SDS-PAGE and immunoblot analysis as described previously (6), using antibodies that detect phosphorylated/active $ERK_{1/2}$ and total $ERK_{1/2}$. Quantitation of the band intensities was done as indicated previously for the Src kinase assay. For each ERK band, the ratio of activated ERK measured with one antibody to total ERK measured with the other was calculated. This ratio was expressed as unity for the unstimulated control sample, and the ratio for any other sample was expressed relative to the corresponding control value. We have shown previously (6) that ouabain-induced activation of $ERK_{1/2}$ measured by this standard procedure agrees with that measured by the in-gel assay of $ERK_{1/2}$ activity.

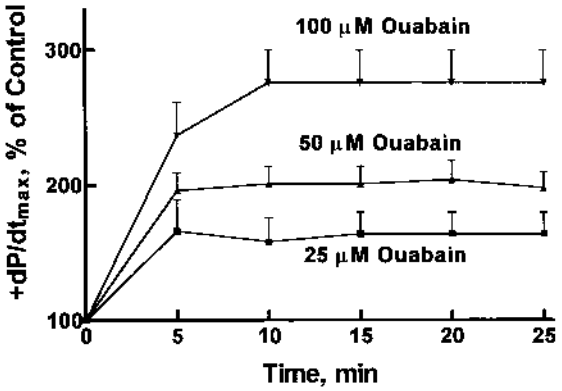

FIG. 1. Time-course of the positive inotropic effects of varying ouabain concentrations on the isolated rat heart. Experiments on Langendorff preparations were done as described in Methods. At each time point, +dp/dt$_{max}$ is expressed relative to that of the control untreated heart. All indicated values (mean ± SE) were significantly higher than controls ($P < 0.001$); n = 6 hearts.

Materials

Antibodies against ERK$_{1/2}$ (K-23), phosphorylated ERK$_{1/2}$ (E-4), and c-Src (polyclonal SRC-2) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The monoclonal antibody against Src (clone GD11), the Src kinase assay kit, and Protein G agarose beads were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Dioleyl-sn-glycerol and phosphatidylserine were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.), and other chemicals from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Since our previous studies on the signal transducing function of Na^+/K^+-ATPase were done on cultured rat cardiac myocytes, we began the present work using the isolated rat heart. Figure 1 shows the results of experiments on the time-course of the effects of three different ouabain concentrations, in the range of 25–100 μ*M*, on +dp/dt$_{max}$. Clearly, dose-dependent positive inotropic effects of ouabain were obtained that reached their peaks within 5–10 min, and were sustained for 25 min. These results are in close agreement with previous observations on the dose-dependent positive inotropic effects of ouabain on isolated rat heart Langendorff preparations (15,16) and isolated rat ventricular strips (17,18). When 5 μ*M* ouabain was used in experiments similar to those shown in Figure 1, an increase in +dp/dt$_{max}$ (120%–130% of control) was noted in some hearts, but no such increase was obtained in most hearts. At the highest ouabain concentration used (100 μ*M*), arrhythmias were obtained in some, but not all, hearts. No evidence of such toxicity was noted at the lower ouabain concentrations used. Based on these results, we chose to do most of the following experiments on the rat heart using 50 μ*M* ouabain.

To determine if ouabain-induced signaling accompanies the positive inotropic action of ouabain in the isolated rat heart, we focused on the activation of Src kinase, PKC, and ERK$_{1/2}$, because in cultured myocytes these sequential signaling events are detected rapidly within 1–20 min after exposure to ouabain (6,7,10). Langendorff preparations that were exposed to 50 μ*M* ouabain for 10 min and the unexposed controls were frozen rapidly, and the appropriate lysates were used for the indicated assays. The results (Fig. 2) show that ouabain caused activation of Src kinase comparable to that noted in cultured neonatal rat cardiac myocytes (7). Stimulus-induced activation of PKC in the intact cell involves the translocation of the enzyme from the cytosolic to the particulate cellular pools; and in cultured cardiac myocytes ouabain induces such translocation/activation (10). The data of Figure 2 show that 50 μ*M* ouabain also caused a significant increase in the PKC activities of the particulate fractions of the Langendorff heart lysates. The apparent reduction in the cytosolic PKC of the ouabain-treated hearts (Fig. 2) was not significant. This finding is not surprising since cytosolic PKC activity constitutes more than 90% of the total cellular activity (10); hence, the noted 30–40% ouabain-induced increase in the activity of the particulate fraction (Fig. 2) would be expected to cause only about a 3% or 4% decrease in the activity of the cytosolic fraction. The data of Figure 3 clearly show that ERK$_{1/2}$ was also activated significantly in isolated rat hearts exposed to 50 μ*M* ouabain. In experiments similar to those shown in Figures 2 and 3, lower ouabain concentrations (1–5 μ*M*) did not cause significant in-

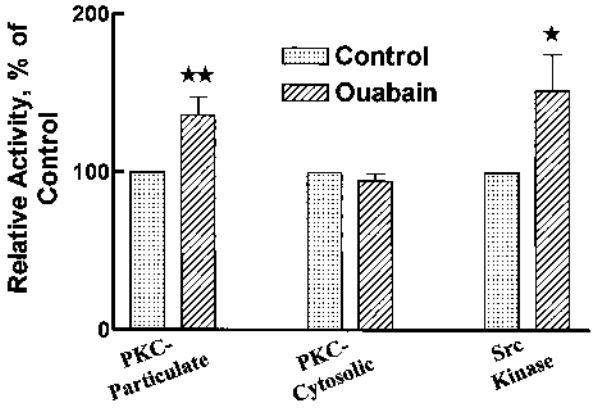

FIG. 2. Ouabain-induced activation of PKC and Src kinase in the isolated rat heart. Langendorff hearts were exposed to 50 μ*M* ouabain for 10 min as in Figure 1. Lysates from the treated and control hearts were prepared and assayed for the indicated activities as described in Methods. Values are mean ± SE; n = 6 hearts. *$P < 0.05$; **$P < 0.01$.

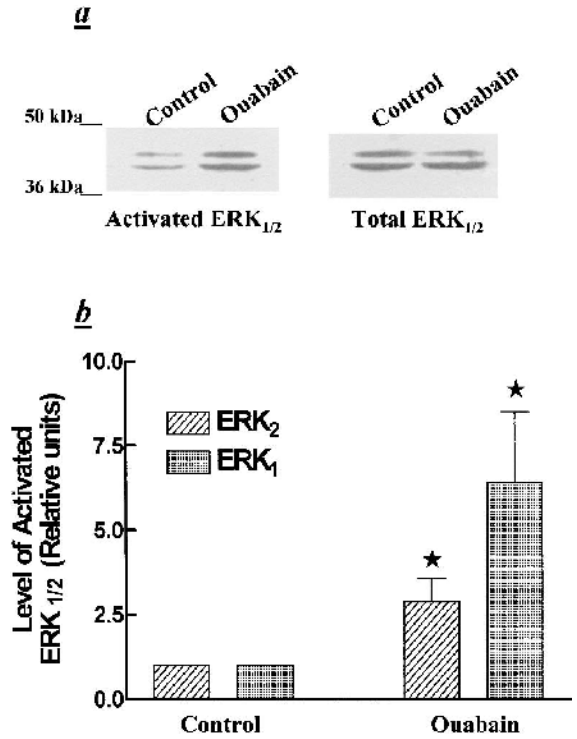

FIG. 3. Ouabain-induced activation of $ERK_{1/2}$ in the isolated rat heart. Langendorff hearts were exposed to 50 μ*M* ouabain for 10 min as in Figure 1. Lysates were immunoassayed for total $ERK_{1/2}$ and activated $ERK_{1/2}$, and activation of each ERK was determined relative to that of control as described in Methods. **A.** Representative blots of ERK_1 (44 kd) and ERK_2 (42 kd). Positions of the nearest molecular weight markers are shown on the left. **B.** Values (mean ± SE) calculated from the scans of multiple blots. n ≥ 6 hearts; **P* < 0.05.

creases in protein kinase activities (data not shown). Taken together, these findings indicate that in the isolated rat heart rapid activations of Src, PKC, and $ERK_{1/2}$ are obtained in response to an ouabain concentration that produces positive inotropy without toxicity, but not in response to lower ouabain concentrations that have little or no effect on contractility.

To test the possibility that these effects of ouabain may be limited to the rat heart, we conducted similar experiments using guinea pig hearts. Previous comparisons between ouabain's positive inotropic effects and inhibitory effects on Na^+/K^+-ATPase activities in cardiac preparations from rats and guinea pigs have shown that the latter is about one or two orders of magnitude more sensitive to ouabain (18,19). In preliminary experiments similar to those shown in Figure 1 on the positive inotropic effect of ouabain on the isolated guinea pig heart, we confirmed that this preparation was about 50-fold more sensitive than the corresponding rat heart preparation. Therefore, we chose a 1-μ*M* ouabain concentration to conduct the experiments, the results of which are shown in Figure 4. The increase in the contractility of the guinea pig heart induced by 1 μ*M* ouabain (Fig. 4A) was accompanied by significant activations of $ERK_{1/2}$ (Figs. 4B and 4C). The potential functional significance of the different patterns of $ERK_{1/2}$ activations in the guinea pig (Fig. 4C) and rat (Fig. 3B) heart is not known.

DISCUSSION

The ouabain-induced signal pathways that have been shown to link Na^+/K^+-ATPase to $ERK_{1/2}$ in cultured rat

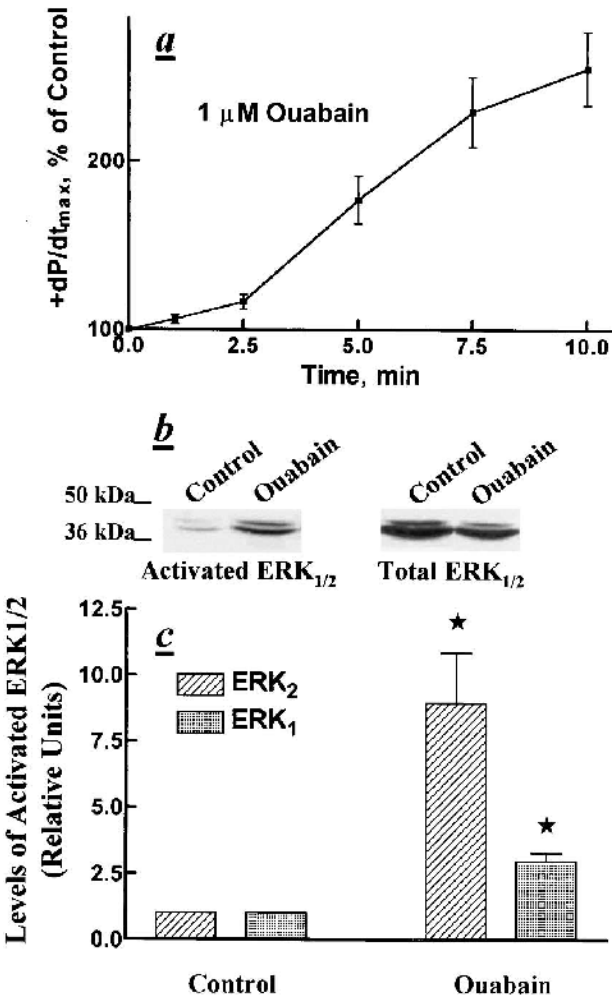

FIG. 4. The positive inotropic effect of ouabain and the accompanied activation of $ERK_{1/2}$ in the isolated guinea pig heart. Langendorff hearts were exposed to 1 μ*M* ouabain, and assayed for contractility and $ERK_{1/2}$ as described in Methods. **A.** Time-course of the ouabain-induced increase in $+dp/dt_{max}$. **B.** Representative immunoblots of ERK_1 (44 kd) and ERK_2 (42 kd). Positions of the nearest molecular weight markers are shown on the left. **C.** Activation of $ERK_{1/2}$ (mean ± SE) calculated from multiple blots. n = 4 hearts; **P* < 0.05.

cardiac myocytes (6,7,10) are summarized in Figure 5. The present findings clearly show that in the course of the development of ouabain-induced positive inotropy in the isolated rat heart, activations of $ERK_{1/2}$ and two key intermediate protein kinases of the pathways leading to $ERK_{1/2}$ are also observed. It is reasonable to conclude, therefore, that the activation of the entire cascade of events depicted in Figure 5 indeed accompanies the positive inotropic action of ouabain on the intact heart, ruling out the possibility that the similar previously observed ouabain-induced signaling events in myocytes were artifacts of the dispersion and culturing of the cardiac myocytes.

The other main finding reported here is that ouabain-induced activation of $ERK_{1/2}$ is also obtained in the isolated guinea pig heart (Fig. 4), and that the different ouabain sensitivities of $ERK_{1/2}$ activation in the hearts of guinea pigs and rats correspond to the established differences in the ouabain sensitivities of the Na^+/K^+-ATPase activities and the contractilities in the heart (18,19). This rules out the possibility that the ouabain-induced signaling events depicted in Figure 5 are a species-specific peculiarity of the rat heart, or due to the well-known relative insensitivity of the rat heart to cardiac glycosides (2,3). It is reasonable, therefore, to assume that the positive inotropic effects of cardiac glycosides in the hearts of other species are also accompanied by the activation of the indicated signaling pathways emanating from the cardiac Na^+/K^+-ATPase.

In cultured cardiac myocytes, the proximal ouabain-induced pathways depicted in Figure 5 have been shown to lead to downstream pathways and consequences that include 1) *ras*-dependent increase in mitochondrial generation of reactive oxygen species (20); 2) activation of transcription factors AP-1 and NF-κB (4,20); 3) induction or repression of several growth-related genes (4–6); and 4) reactive oxygen species-dependent increase in protein synthesis and hypertrophic growth (20). The present demonstration of the similarities of the proximal pathways of Figure 5 in the cultured cardiac myocytes and the intact heart does not guarantee the same degree of similarity in the downstream consequences. In fact, it is safe to assume that in the intact animal, where the extensive network of the signal transduction pathways emanating from cardiac Na^+/K^+-ATPase (11) must interact with related and overlapping networks induced by other stimuli or receptors (21), the downstream cardiac consequences of the ouabain-induced signaling must certainly differ in some respects from those noted either in cultured myocytes or in isolated heart preparations. While the clarification of the nature and the extent of these differences must await the outcomes of future studies, we suggest that the present findings are sufficient to indicate that the consequences of chronic exposure of the heart to the positive inotropic concentrations of cardiac glycosides must extend well beyond the established increase in the force of contraction. Involvement of Src, PKC, and $ERK_{1/2}$ in the development of cardiac hypertrophy and/or failure in the intact heart is now well established (21–24). Based on this, and the general knowledge of the regulatory roles of these protein kinases, we suggest that the mere demonstration of the ouabain-induced activation of these kinases in the isolated heart strengthens the proposition that the consequences of chronic treatment with cardiac glycosides must include some drug-induced changes in the cardiac phenotype, though not necessarily in cardiac growth. Whether these changes are beneficial or harmful is critical to the resolution of the long-standing controversies on the risk/benefit ratio of the use of cardiac glycosides for the treatment of heart failure. These controversies persist due to the ambiguities of recent clinical trials designed to answer these questions (25). Thus, in spite of the well-established efficacies of more modern drugs, the current international guidelines on the management of the heart failure still include treatment with cardiac glycosides (26,27). We suggest the necessity of further studies on cardiac glycoside-induced signal transduction through Na^+/K^+-ATPase for the determination of the rightful place of these venerable drugs in modern therapeutics.

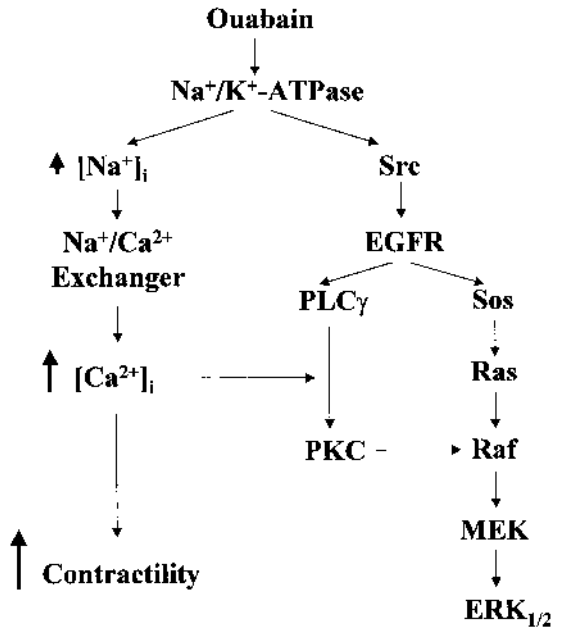

FIG. 5. Ouabain-induced signal pathways that link cardiac Na^+/K^+-ATPase to $ERK_{1/2}$.

CONCLUSION

Evidently, the early events in the newly appreciated signal transducing pathways that are elicited in cultured cardiac myocytes due to interactions of cardiac glycosides

with Na^+/K^+-ATPase also accompany the classic effects of these drugs on the contractility of the isolated intact heart. The consequences of the activations of these signal pathways in the heart of the intact animal or a patient treated with these drugs remain to be explored.

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