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Role of Protein Kinase C in the Signal Pathways That Link Na^+/K^+-ATPase to ERK1/2*

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Kamiar Mohammadi, Peter Kometiani, Zijian Xie, and Amir Askari‡

From the Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43614

We have shown before that Na^+/K^+-ATPase acts as a signal transducer, through protein-protein interactions, in addition to being an ion pump. Interaction of ouabain with the enzyme of the intact cells causes activation of Src, transactivation of EGFR, and activation of the Ras/ERK1/2 cascade. To determine the role of protein kinase C (PKC) in this pathway, neonatal rat cardiac myocytes were exposed to ouabain and assayed for translocation/activation of PKC from cytosolic to particulate fractions. Ouabain caused rapid and sustained stimulation of this translocation, evidenced by the assay of Ca^{2+}-dependent and Ca^{2+}-independent PKC activities and by the immunoblot analysis of the α, δ, and ϵ isoforms of PKC. Dose-dependent stimulation of PKC translocation by ouabain (1–100 μM) was accompanied by no more than 50% inhibition of Na^+/K^+-ATPase and doubling of $[Ca^{2+}]_i$, changes that do not affect myocyte viability and are known to be associated with positive inotropic, but not toxic, effects of ouabain in rat cardiac ventricles. Ouabain-induced activation of ERK1/2 was blocked by PKC inhibitors calphostin C and chelerythrine. An inhibitor of phosphoinositide turnover in myocytes also antagonized ouabain-induced PKC translocation and ERK1/2 activation. These and previous findings indicate that ouabain-induced activation of PKC and Ras, each linked to Na^+/K^+-ATPase through Src/EGFR, are both required for the activation of ERK1/2. Ouabain-induced PKC translocation and ERK1/2 activation were dependent on the presence of Ca^{2+} in the medium, suggesting that the signal-transducing and ion-pumping functions of Na^+/K^+-ATPase cooperate in activation of these protein kinases and the resulting regulation of contractility and growth of the cardiac myocyte.

Na^+/K^+-ATPase is the intrinsic enzyme of the plasma membrane that maintains the normal gradients of Na^+ and K^+ across this membrane of most animal cells (1, 2). In recent years, we have shown that Na^+/K^+-ATPase also acts as a signal transducer; *i.e.* it responds to extracellular stimuli such as ouabain or low extracellular K^+ to relay messages, through protein-protein interactions and second messengers, to intracellular signaling complexes, the mitochondria, and the nucleus (3–9). The interaction of Na^+/K^+-ATPase with nontoxic concentrations of the digitalis drug ouabain causes the activation of multiple interrelated signal pathways that seem to begin with the activation of Src kinase, followed by Src-induced transactivation of EGFR,[1] recruitment and activation of Ras and activation of two Ras-dependent branched pathways: one communicating with the mitochondria to increase the generation of mitochondrial reactive oxygen species and the other being the Ras/Raf/MEK/ERK1/2 cascade (3–9). Although we have demonstrated the ouabain-induced activation of these pathways in several cell types (8, 9), to date the downstream consequences have only been examined in cardiac myocytes (3–9). In these cells, where the well established inhibitory effects of nontoxic ouabain concentrations on the ion-pumping function of Na^+/K^+-ATPase lead to small increases in $[Na^+]_i$ and significant increases in $[Ca^{2+}]_i$, this rise in $[Ca^{2+}]_i$ cooperates with the Ca^{2+}-independent activation of the mitochondrial reactive oxygen species to regulate the transcription of growth-related genes and cause myocyte hypertrophy (9). A question raised in the course of these studies on cardiac myocytes was on the possible role of PKC in the proximal pathways that are activated by ouabain. Our early experiments showed that inhibition of PKC blocked the ouabain-induced regulation of several growth-related genes (3, 4). Since PKC inhibition also seemed to antagonize the rapid ouabain-induced activation of ERK1/2 (6), we suggested that at least one locus of PKC involvement must be upstream of ERK1/2 (6). This, coupled with the fact that direct activation of PKC by PMA causes activation of ERK1/2 in myocytes (6, 10), led to the postulate that ouabain may indeed cause the rapid activation of PKC in cardiac myocytes. The present studies were initiated to test this hypothesis and to explore the mechanisms of any ouabain-induced PKC activation and its linkage to activation of ERK1/2.

EXPERIMENTAL PROCEDURES

Materials—Chemicals of highest purity and culture media were purchased from Sigma. Antibodies against PKCα (H-7), PKCϵ (C-15), PKCδ (C-17), ERK1/2 (K-23), and phosphorylated ERK1/2 (E-4) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Calphostin C and chelerythrine were purchased from Calbiochem, D609 from Sigma, phosphatidylserine and dioleyl-*sn*-glycerol from Avanti Polar Lipids (Alabaster, AL), Epep from Quality Control Biochemicals (Hopkinton, MA), and fura-2 and fura-2/AM from Molecular Probes, Inc. (Eugene, OR).

Cell Preparation and Culture—The same protocols were used to prepare and culture neonatal ventricular myocytes as described before (3). In short, myocytes from 1-day-old Harlan Sprague-Dawley rats were isolated and purified on Percoll gradients. In a medium containing four parts of Dulbecco's modified Eagle's medium and one part Medium

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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 and trival name used are: EGFR, epidermal growth factor receptor; D609, tricyclodecan-9-yl-xanthogenate; Epep, PKCϵ substrate peptide; ERK, extracellular signal-regulated kinase/mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PKC, protein kinase C; PLC, phospholipase C; RACK·PKC, receptor for activated PKC; PMA, phorbol 12-myristate 13-acetate; Raf, Raf-1 kinase.

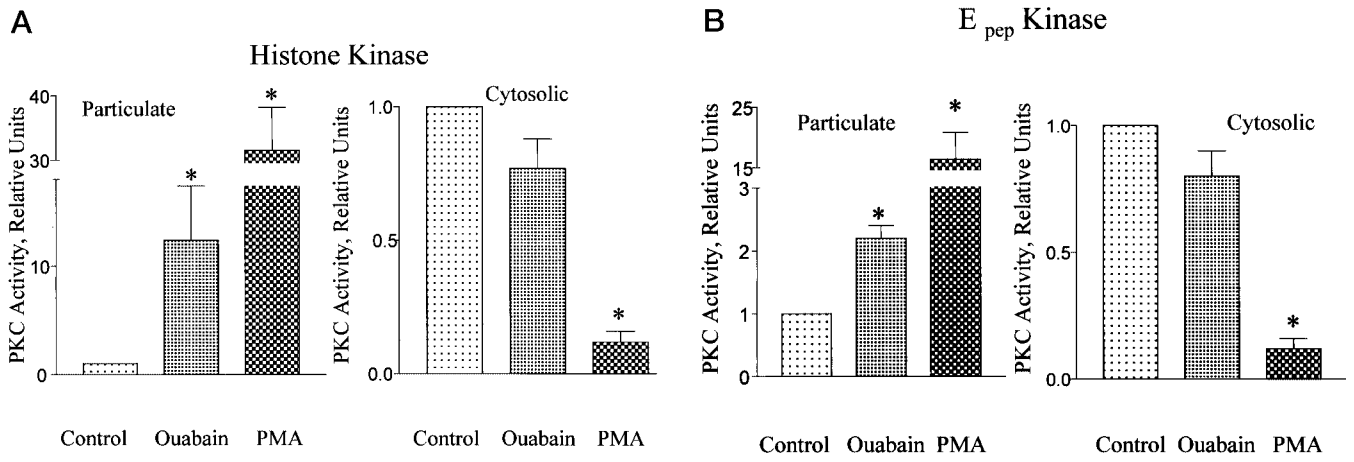

FIG. 1. **Effects of ouabain and PMA on the translocation of PKC from cytosolic to particulate fractions of myocytes.** *A*, Ca^{2+}-stimulated PKC activity (histone kinase). *B*, Ca^{2+}-independent PKC activity (Epep kinase). Cells were exposed to 100 μM ouabain for 10 min or 100 nM PMA for 5 min, lysed, and assayed for the indicated activities as described under "Experimental Procedures." Control activities were as follows: particulate histone kinase, 3.2 ± 1.6 pmol/μg/min; cytosolic histone kinase, 132 ± 22 pmol/μg/min; particulate Epep kinase, 14.7 ± 3.8 pmol/μg/min; cytosolic Epep kinase, 219 ± 33 pmol/μg/min. Activity of each ouabain- or PMA-treated sample was compared with the corresponding control. $n \geq 6$; *, $p < 0.05$.

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 Dulbecco's modified Eagle's medium 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 (3). Immunofluorescence staining with a myosin heavy chain antibody showed that the cultures contained more than 95% myocytes.

Assay of PKC Activity and Translocation—Myocytes were washed with phosphate-buffered saline; suspended in a solution containing 10 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 25 μg/ml aprotinin, and 20 mM Tris-HCl (pH 7.5); and homogenized in a Potter-Elvehjen homogenizer. The suspension was centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was removed (the cytosolic fraction), and the pellet was suspended in the above described solution to which Triton X-100 (1%) was also added. After 30 min on ice, the mixture was centrifuged at 25,000 × *g* for 10 min, and the supernatant was collected (the particulate fraction).

PKC activities were determined by slight modifications of previously described procedures (13–16). Briefly, the cytosolic and the particulate fractions (above) were partially purified by passage through DE-52 cellulose columns (15, 16) and assayed either for Ca^{2+}-stimulated PKC activity using histone H_1 as substrate (histone kinase) or for Ca^{2+}-independent PKC activity using the synthetic substrate of PKC_ϵ, Epep (Epep kinase). The reaction mixtures contained 20 mM Tris-HCl (pH 7.5), 10 μM [γ-^{32}P]ATP, 10 mM magnesium acetate, 0.75 mM $CaCl_2$, 50 μg/ml leupeptin, either 100 μg/ml histone or 50 μg/ml Epep, with or without 30 μM phosphatidylserine, and 0.5 μM dioleyl-*sn*-glycerol. After incubation at 30 °C, reactions were stopped by spotting aliquots on phosphocellulose paper (P81) and washing each paper three times with 25 ml of 0.75% phosphoric acid. The collected ^{32}P-labeled substrate was counted by conventional procedures. Reaction time and enzyme concentrations were chosen to ensure the assay of initial velocities.

To assay the translocation of the various PKC isoforms, the cytosolic and the particulate fractions were subjected to immunoblot analysis, using the indicated isoform-specific antibodies and appropriate secondary antibodies, as described before (15, 16). Quantitation of the relative intensities of the immunoblots were done using chemiluminescence and exposure to x-ray film. Images were scanned with a Bio-Rad densitometer. When necessary, different dilutions of the samples were subjected to immunoblotting, and multiple exposures of the films were used to ensure that quantitative comparisons were made within the linear range of the assay.

Measurement of Phosphorylation/Activation of ERK1/2—After the indicated treatments, cells were washed with phosphate-buffered saline and lysed in an ice-cold solution containing 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM tetrasodium pyrophosphate, 10 nM okadaic acid, 1% Triton X-100, 0.25% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 mM Tris-HCl (pH 7.4). Equal amounts of protein from various lysates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis as described before (6) using antibodies that detect only phosphorylated/active ERK1/2 and total ERK1/2. Activation was measured as the ratio of the intensities of the appropriate bands detected with the two antibodies. We have shown before that ouabain-induced activation of ERK1/2 by this procedure correlates with in-gel assay of ERK1/2 activities (6).

$^{86}Rb^+$ Uptake—The initial rate of ouabain-sensitive Rb^+ uptake through the Na^+/K^+-ATPase of intact myocytes was measured as described before (17), using monensin in the assay medium to ensure that the maximal capacity of the active uptake was being measured (18). Briefly, myocytes were incubated in the culture medium at 37 °C for 10 min with different concentrations of ouabain, including the maximally effective 1 mM ouabain; 25 μM monensin and $^{86}Rb^+$ as tracer for K^+ were then added, and uptake was measured after 20 min. It was established that uptake was a linear function of time within this period.

Fluorescence Microscopic Assay of $[Ca^{2+}]_i$—This was done using fura-2, by the procedures and calibrations described before (7, 17). Briefly, fura-2 fluorescence was recorded using an Attofluor imaging system (Atto Instruments, Rockville, MD) at excitation wavelengths of 340/380 nm and at an emission wavelength of 505 nm. Under each experimental condition, time-averaged signals were obtained from about 20–40 single cells. $[Ca^{2+}]_i$ was calculated based on the fluorescence ratio and the Ca^{2+} calibration curve (7, 17).

Data Analysis—Means ± S.E. of the results of a minimum of three experiments are presented. Student's *t* test was used, and significance was accepted at $p < 0.05$.

RESULTS

Ouabain-induced Activation of PKC—Stimulus-induced activation of PKC involves the translocation of the enzyme from the soluble to the particulate fractions of cells (19, 20). In experiments of Fig. 1, neonatal rat cardiac myocytes were exposed to 100 μM ouabain for 10 min or 100 nM PMA for 5 min as positive control and were assayed for PKC activities in particulate and cytosolic fractions. In each fraction Ca^{2+}-stimulated PKC activity (histone kinase) and Ca^{2+}-independent PKC activity (Epep kinase) were assayed (Fig. 1, *A* and *B*). As expected, in control unstimulated cells, a small fraction of either histone kinase or Epep kinase was in the particulate fraction (see legend to Fig. 1), and upon PMA stimulation the greater portion of each activity was shifted from the cytosolic to the particulate fraction (Fig. 1). Ouabain also caused significant increases in the particulate fraction activities of histone

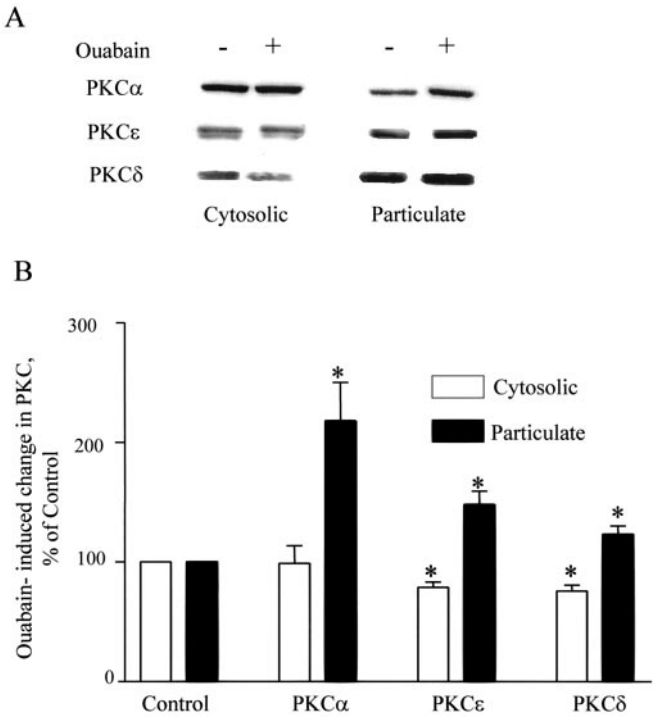

FIG. 2. **Effects of ouabain on the translocation of PKCα, PKCε, and PKCδ.** Myocytes were exposed to ouabain as in Fig. 1. The particulate and the cytosolic fractions were subjected to Western blot analysis as indicated under "Experimental Procedures." *A*, representative blots. *B*, quantitative comparisons of blots from multiple experiments. $n \geq 10$; *, $p < 0.05$.

kinase (Fig. 1*A*) and Epep kinase (Fig. 1*B*); however, these ouabain-induced increases were less than those induced by PMA. The concomitant ouabain-induced decreases in cytosolic activities, which were expected to be small, were not statistically significant (Fig. 1).

To determine the effects of ouabain on individual PKC isoforms, we focused on α, ε, and δ isoforms whose translocation from the cytosolic to particulate fractions has been shown to be affected by a number of other stimuli in the neonatal rat cardiac myocytes used here (21–25). Exposure of myocytes to 100 μM ouabain for 10 min increased the amount of each isoform, assayed with isoform-specific antibodies, in the particulate fraction (Fig. 2). In the cytosolic fraction, significant ouabain-induced decreases in the levels of immunoreactive ε and δ isoforms, but not in that of α isoform, were also detected (Fig. 2). When the time course of the effects of 100 μM ouabain on the translocation of the three isoforms to the particulate fraction were examined, maximal increases were noted as early as 1 min after exposure to ouabain, and the increases were sustained for at least 20 min (Fig. 3).

The results of the experiments of Fig. 4A on the dose dependence of the ouabain-induced translocation of PKCα to the particulate fraction showed the effectiveness of ouabain concentrations as low as 1 μM. To allow the direct comparison of these effects with dose-dependent ouabain effects on the ion transport function of the Na^+/K^+-ATPase of these myocytes, the effects of selected ouabain concentrations on active Rb^+ uptake and on $[Ca^{2+}]_i$ of the intact myocytes were also determined (Fig. 4, *B* and *C*). The highest concentration of ouabain used in experiments on PKC (100 μM) caused about 50% inhibition of the maximal pumping capacity of Na^+/K^+-ATPase (Fig. 4*B*) and about doubling of the $[Ca^{2+}]_i$ (Fig. 4*C*). Clearly, ouabain-induced PKC activation/translocation is obtained in the absence of toxic Ca^{2+} overload. This is in agreement with our previous data (3) showing no significant change in the viability of these myocytes after 12 h of exposure to 100 μM ouabain.

Role of PLC in Ouabain-induced Activation of PKC—Because activations of polypeptide growth factor receptors such as EGFR are known to stimulate phosphoinositide turnover by activation of PLC-γ (26) and since ouabain transactivates EGFR in the myocytes used here (8), it was reasonable to suspect that the above ouabain-induced activation of PKC might be due to stimulation of phosphoinositide turnover. In fact, ouabain-induced increases in inositol phosphates and diacylglycerol in several cell types, including cardiac preparations, have been noted before (27–30), albeit at ouabain concentrations that are expected to be toxic to cardiac myocytes. To determine whether the ouabain-induced activation/translocation of PKC noted here was indeed linked to stimulation of phosphoinositide turnover, we used a PLC inhibitor, D609. Although this compound is a more selective inhibitor of phosphatidylcholine-specific PLC in several cell types (31, 32), it has been shown to inhibit phosphatidylinositol-specific PLC in the intact neonatal rat cardiac myocytes (24, 33, 34). Pretreatment of myocytes with D609 blocked ouabain-induced translocation of PKCα and PKCε to the particulate fraction (Fig. 5), but it had no significant effect on PMA-induced translocation of the two isoforms (Fig. 6). This and the fact that D609 also had no effect on PMA-induced activation of histone kinase and Epep kinase (data not shown) ruled out the possibility that D609 might have a direct inhibitory effect on PKC. Taken together, the above data support the proposition that ouabain-induced activation/translocation of PKC is the consequence of PLC activation linked to the ouabain-induced transactivation of EGFR.

Relationship between Ouabain-induced Activations of PKC and ERK1/2—We showed before (6) that a rather nonspecific PKC inhibitor, H-7, antagonized ouabain-induced activation of ERK1/2. This was confirmed with the use of more specific PKC inhibitors chelerythrine and calphostin C (Fig. 7). D609 also blocked ouabain-induced activation of ERK1/2 (Fig. 7), indicating that sequential activations of PLC and PKC are required for ouabain-induced activation of ERK1/2.

In previous studies, we showed that ouabain-induced activation of ERK1/2 also required the presence of extracellular Ca^{2+} (6). The question arose as to whether this dependence on extracellular Ca^{2+} was exerted upstream or downstream of PKC activation. In myocytes that were incubated in a nominally Ca^{2+}-free medium, exposure to ouabain did not activate PKC in contrast to the activation noted in the Ca^{2+}-containing medium (Fig. 8). Evidently, the well established ouabain-induced net influx of Ca^{2+} that leads to a rise in $[Ca^{2+}]_i$ (Fig. 4) is required for ouabain-induced activations of PKC and ERK1/2.

DISCUSSION

A main new finding reported here is that ouabain interaction with intact cardiac myocytes leads to rapid translocation/activation of several PKC isoforms from the soluble to the particulate pools of these enzymes (Figs. 1–3). Before we discuss the implications of these and related findings, it is important to emphasize that the data presented here (Fig. 4), in conjunction with previous findings, clearly indicate that the activating effect of ouabain on PKC is obtained at ouabain concentrations that cause only partial inhibition of Na^+/K^+-ATPase (Fig. 4*B*), small changes in $[Na^+]_i$ (9), and significant but nontoxic increases in $[Ca^{2+}]_i$ (Fig. 4*B*; Refs. 3 and 9). Such degrees of change in Na^+/K^+-ATPase and intracellular cation concentrations are indeed the requirements for obtaining the positive inotropic effects of ouabain and related digitalis drugs (9, 35, 36). In fact, it has been shown (35, 37) that the major portion of the positive inotropic action of ouabain on rat ventricular strips

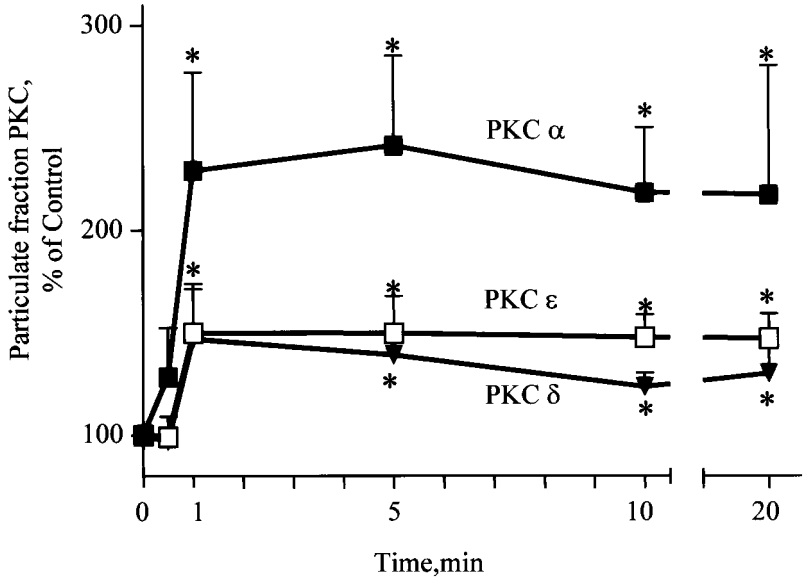

FIG. 3. **Time course of the ouabain-induced increase in immunoreactive PKC isoforms in the particulate fraction.** Myocytes were exposed to 100 μM ouabain, and the assays were done as in Fig. 2. $n \geq 3$; *, $p < 0.05$.

is obtained at ouabain concentrations (10–100 μM) comparable with those used in the present studies. Thus, although ouabain effect on the contractile strength of the cultured myocytes was not measured here,[2] it is reasonable to conclude that the present findings suggest that ouabain-induced PKC activation must accompany the classical effect of ouabain on cardiac contractility, at least in the rat.

Activations of both PKC and Ras Are Required for the Linkage of Na^+/K^+-ATPase to ERK1/2—The present findings provide strong support for the previous suggestion (6) that ouabain-induced PKC activation precedes the activation of ERK1/2. To date, the most proximal signaling events that have been identified to be linked to Na^+/K^+-ATPase are the activation of Src and the transactivation of EGFR (8). Evidently, these events lead not only to the activation of Ras (6, 8) but also to the parallel activation of PLC, stimulation of the phosphoinositide turnover, and activation of PKC (Fig. 5). That this PKC activation is essential for ouabain-induced activation of ERK1/2 (Fig. 7) must now be reconciled with our previous data showing that activation of Ras is also essential for ouabain-induced activation of ERK1/2 (6). A reasonable hypothesis is that Ras and PKC must cooperate to activate Raf. This is supported by observations in cells other than cardiac myocytes (38–39), indicating that Raf recruitment to the plasma membrane requires activated Ras but that activation of the recruited Raf requires additional steps that include phosphorylation of Raf, possibly by PKC. Based on the present data and our previous findings, the status of the signal pathway that links Na^+/K^+-ATPase to ERK1/2 through PKC and Ras is summarized in Fig. 9. It is important to point out, however, that the steps outlined in Fig. 9 may not be the only links between Na^+/K^+-ATPase and PKC. In view of the recent evidence suggesting the interaction of Src with RACK·PKC complexes (40, 41), it is possible that ouabain-induced activated Src may also be involved in

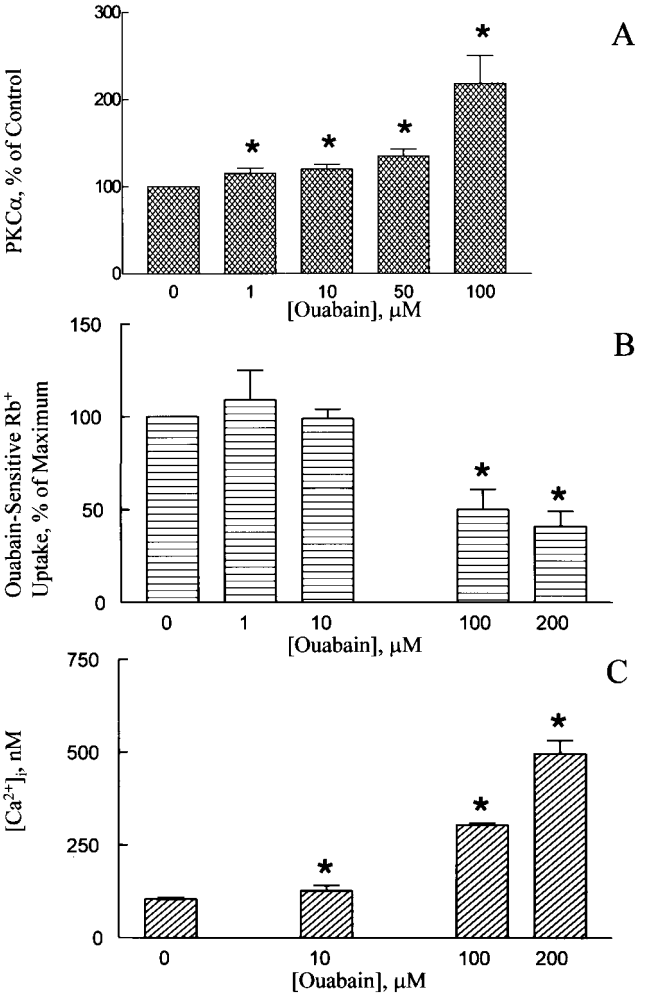

FIG. 4. **Comparison of the effects of varying ouabain concentrations on PKCα translocation to the particulate fraction (*A*), active uptake of Rb^+ (*B*), and $[Ca^{2+}]_i$ (*C*).** Immunoblots of PKCα were done as in Fig. 3 ($n \geq 3$). Ouabain effects on $^{86}Rb^+$ uptake were assayed as described under "Experimental Procedures." At each indicated ouabain concentration, uptake is expressed as percentage of the maximal uptake inhibited by 1 mM ouabain ($n \geq 6$). The $[Ca^{2+}]_i$ values, determined as indicated under "Experimental Procedures," are the time-averaged means of values in a minimum of 28 cells after 15–20 min of exposure to ouabain. *, $p < 0.05$.

[2] The low density, serum-starved myocyte cultures used here consist of predominantly quiescent cells (*i.e.* most cells do not contract spontaneously, and some contract at a low rate). As indicated before (9), the easily noted ouabain effects in these cultures are increases in the number of contracting cells and the rate of contractions. Under these circumstances, it is difficult to quantitate the effect of ouabain on the contractile force (cell shortening) or Ca^{2+} transients. We have shown elsewhere (42), however, that in electrically paced cultured myocytes, ouabain increases systolic $[Ca^{2+}]_i$, diastolic $[Ca^{2+}]_i$, and the time-averaged $[Ca^{2+}]_i$, as expected.

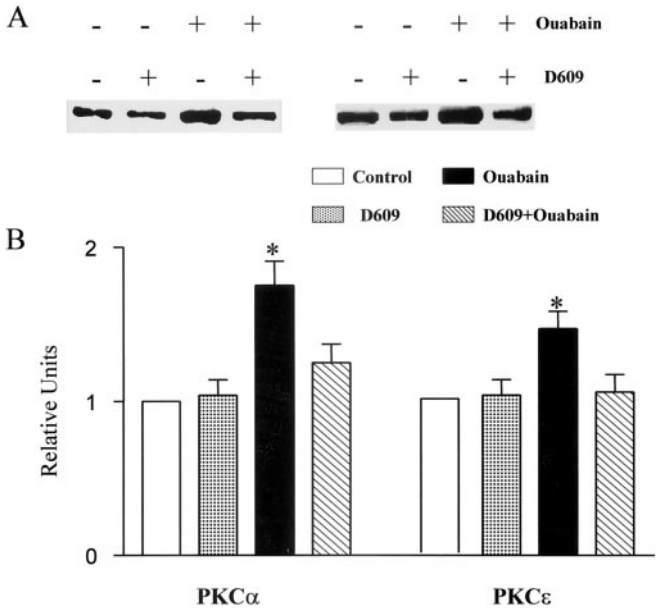

FIG. 5. **Inhibition of ouabain-induced translocation of PKC isoforms by D609.** Effects of 100 μM ouabain on control myocytes and those pretreated with 100 μM D609, a PLC inhibitor, for 12 h were determined as indicated in Fig. 2. *A*, representative blots; *B*, quantitative comparison of blots from multiple experiments. Each value is expressed relative to that of the corresponding control. $n \geq 5$; *, $p < 0.05$.

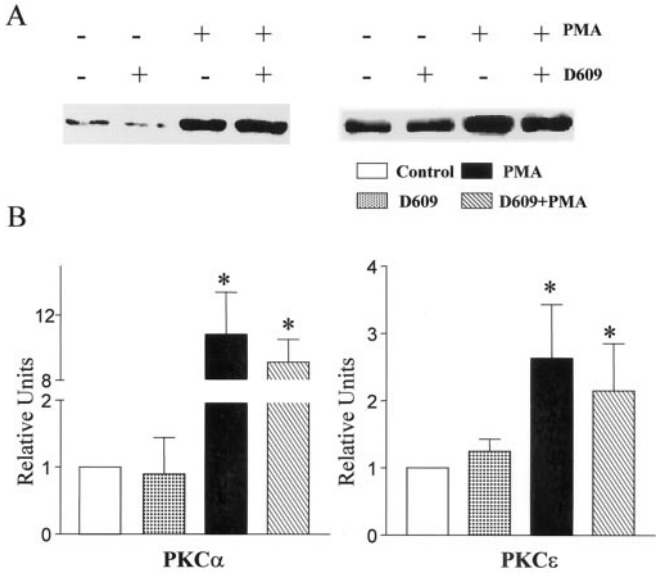

FIG. 6. **Lack of effect of D609 on PMA-induced translocation of the PKC isoforms.** Experiments were done with 100 nM PMA as indicated in Fig. 5. $n \geq 3$; *, $p < 0.05$.

directing the activated PKC isoforms to their substrates, including Na^+/K^+-ATPase itself (see below).

Necessity of Ouabain-induced Rise in $[Ca^{2+}]_i$ for Activations of PKC and ERK1/2—We showed before (9) that activation by ouabain of Src-induced tyrosine phosphorylation of a number of proteins, and Ras-dependent increase in mitochondrial generation of reactive oxygen species were independent of the presence of extracellular Ca^{2+} and ouabain-induced rise in $[Ca^{2+}]_i$. We also showed, however, that ouabain-induced activation of ERK1/2 required the presence of extracellular Ca^{2+} (6). This effect of Ca^{2+} is now shown to be, at least in part, due to the dependence of ouabain-induced PKC activation on extracellular Ca^{2+} (Fig. 8), which is the source of the ouabain-induced rise in $[Ca^{2+}]_i$. Although the precise mechanism of this Ca^{2+}

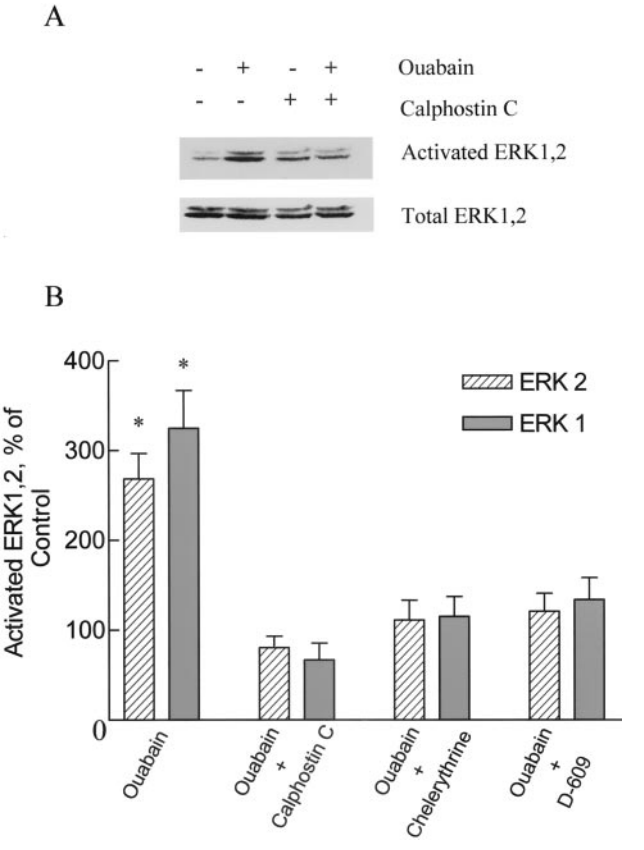

FIG. 7. **Antagonism of the ouabain-induced activation of ERK1/2 by calphostin C, chelerythrine, and D609.** Control myocytes and those pretreated with 0.1 μM calphostin C (15 min), 5 μM chelerythrine (30 min), and 100 μM D609 (12 h) were treated with 100 μM ouabain for 5 min and assayed for activations of ERK1/2 as indicated under "Experimental Procedures." *A*, representative blots. *B*, comparisons of blots from multiple experiments. The value of each ouabain-treated sample is expressed as a percentage of the respective control not treated with ouabain. $n \geq 4$; *, $p < 0.05$.

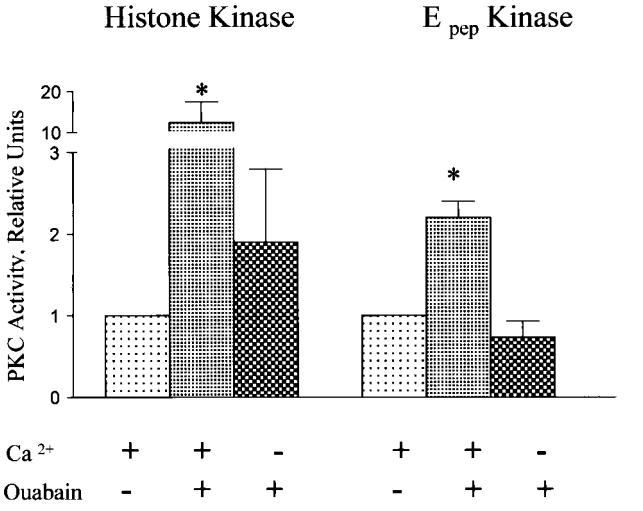

FIG. 8. **Effect of medium Ca^{2+} on ouabain-induced translocation of PKC.** Myocytes incubated in the standard Ca^{2+}-containing culture medium or the Ca^{2+}-free culture medium were exposed to 100 μM ouabain as in Fig. 1 and assayed for histone kinase and Epep kinase activities of the particulate fractions. $n \geq 4$; *, $p < 0.05$.

effect (perhaps on PLC) remains to be determined, the present findings reinforce the conclusion that there is cross-talk between the ion-pumping function and the signal-transducing function of Na^+/K^+-ATPase (Fig. 9).

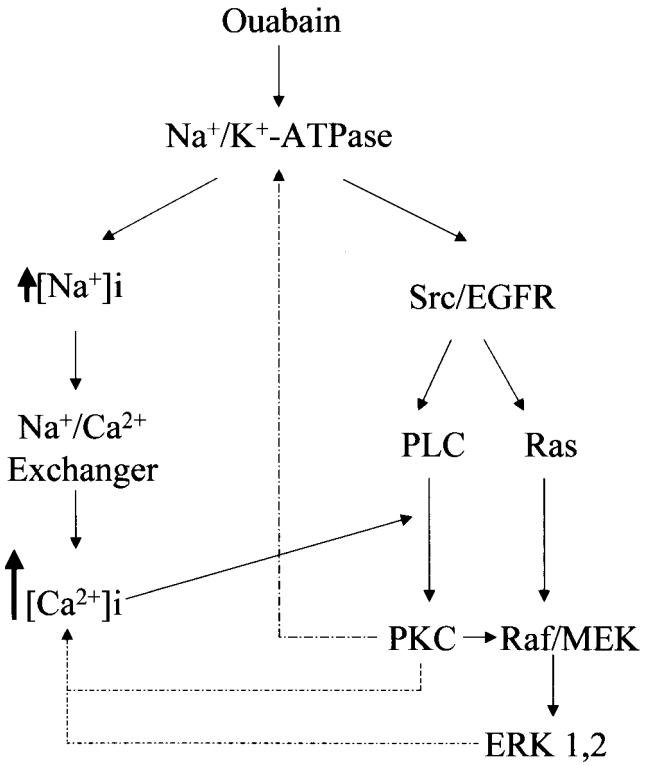

FIG. 9. **Schematic representation of the pathways involved in ouabain-induced activations of PKC and ERK1/2 in cardiac myocytes.** Ouabain induces an inhibited pool of Na^+/K^+-ATPase to communicate with PKC and ERK1/2 through protein-protein interactions (*right*). Reduction of the ion pumping pool of Na^+/K^+-ATPase by ouabain leads to rise in $[Ca^{2+}]_i$ (*left*), which mediates cross-talk between the pathways linked to the two pools. *Solid arrows* indicate events that are supported by experimental evidence presented here or before. The broken arrows are the postulated feedback loops; evidence for those involving PKC is indirect, but there is direct evidence (42) for the feedback regulation of $[Ca^{2+}]_i$ by ERK1/2. See "Discussion" for further details.

Further Implications of Ouabain-induced Activations of PKC and ERK1/2—If translocation/activation of some PKC isoforms does indeed accompany the positive inotropic action and the therapeutic use of digitalis, as suggested by the present findings, several further implications are worthy of brief consideration. 1) In view of the above discussed necessity of ouabain-induced increase in $[Ca^{2+}]_i$ for the rapid and sequential activations of PKC and ERK1/2, the question arises as whether the activation of these protein kinases may in turn influence the ouabain-induced change in $[Ca^{2+}]_i$. Evidently, the answer is yes, since Tian *et al*. (42) have shown recently that inhibition of ERK1/2 in adult cardiac myocytes antagonizes ouabain-induced rise in $[Ca^{2+}]_i$, perhaps through an effect of ERK1/2 on Ca^{2+} channels and/or the Na^+/Ca^{2+} exchanger. There is also a large body of evidence to indicate the role of PKC in short term regulation of $[Ca^{2+}]_i$ through activating or inhibitory effects of PKC on voltage-gated Ca^{2+} channels, the sarcoplasmic reticulum Ca^{2+} uptake/release system, and the sarcolemmal Na^+/Ca^{2+} exchanger (25, 43, 44). The possibility that ouabain-induced activation of PKC may affect any of these transporters, coupled with the demonstrated role of ERK1/2 in the regulation of $[Ca^{2+}]_i$ (42), suggests that PKC, ERK1/2, Na^+/K^+-ATPase, and a number of neighboring membrane receptors and transporters are within a signal feedback cycle (Fig. 9). Thus, the mechanism of the positive inotropic action of ouabain and related drugs may be more complex than previously assumed. 2) Of special interest is the possibility that ouabain-induced translocation/activation of PKC may cause the phosphorylation and regulation of Na^+/K^+-ATPase. It has been known for a long time that Na^+/K^+-ATPase is a substrate for PKC (45, 46); however, establishment of the physiological relevance of this has been difficult, at least in part due to isoform- and species-specific structural differences among different Na^+/K^+-ATPases that affect their interactions with PKC (45–48). In the rat cardiac myocytes where the predominant Na^+/K^+-ATPase isoforms are known to be good substrates for PKC (47), it is not unlikely that PKC-induced changes in the activity and/or endocytosis of Na^+/K^+-ATPase (49, 50) may also be a part of the ouabain-induced feedback cycle (Fig. 9). 3) Finally, we may note that a number of previous studies, including those in cultured cells and transgenic mice, have indicated the involvement of various PKC isoforms and ERK1/2 in the development of cardiac hypertrophy and/or failure (15, 16, 25, 51, 52). The present demonstration of the sequential ouabain-induced activations of PKC and ERK1/2, coupled with the previously shown hypertrophic effects of ouabain in cultured myocytes (3, 4), emphasize the need for further studies on the roles of PKC isoforms in the altered phenotype of the digitalis-treated heart.

REFERENCES

1. Skou, J. C., and Esmann, M. (1992) *J. Bioenerg. Biomembr.* **24,** 249–261
2. Lingrel, J. B, and Kuntzweiler, T. (1994) *J. Biol. Chem.* **269,** 19659–19662
3. Peng, M., Huang, L., Xie, Z., Huang, W.-H., and Askari, A. (1996) *J. Biol. Chem.* **271,** 10372–10378
4. Huang, L., Li, H., and Xie, Z. (1997) *J. Mol. Cell. Cardiol.* **29,** 429–437
5. Huang, L., Kometiani, P., and Xie, Z. (1997) *J. Mol. Cell. Cardiol.* **29,** 3157–3167
6. Kometiani, P., Li, J., Gnudi, L., Kahn, B. B., Askari, A., and Xie, Z. (1998) *J. Biol. Chem.* **273,** 15249–15256
7. Xie, Z., Kometiani, P., Liu, J., Li, J., Shapiro, J. I., and Askari, A. (1999) *J. Biol. Chem.* **274,** 19323–19328
8. Haas, M., Askari, A., and Xie, Z. (2000) *J. Biol. Chem.* **275,** 27832–27837
9. Liu, J., Tian, J., Haas, M., Shapiro, J. I., Askari, A., and Xie, Z. (2000) *J. Biol. Chem.* **275,** 27838–27844
10. Bogoyevitch, M. A., Andersson, M. B., Gillespie-Brown, J., Clerk, A., Glennon, P. E., Fuller, S. J., and Sugden, P. H. (1996) *Biochem. J.* **314,** 115–121
11. Mohammadi, K., Xie, Z., and Askari, A. (2000) *Biophys. J.* **78,** 79 (abstr.)
12. Mohammadi, K., Kometiani, P., Xie, Z., and Askari, A. (2001) *FASEB J.* **15,** A443
13. Thomas, P. T., Gopalakrishna, R., and Anderson, W. B. (1987) *Methods Enzymol.* **141,** 399–412
14. Rybin, V., and Steinberg, S. F. (1996) *Circ. Res.* **79,** 388–398
15. Rouet-Benzineb, P., Mohammadi, K., Pérennec, J., Poyard, M., El Houda Bouanani, N., and Crozatier, B. (1996) *Circ. Res.* **79,** 153–161
16. Mohammadi, K., Rouet-Benzineb, P., Laplace, M., and Crozatier, B. (1997) *J. Mol. Cell. Cardiol.* **29,** 1687–1694
17. Kometiani, P., Askari, A., Liu, J., Xie, Z., and Askari, F. K. (2001) *Am. J. Physiol.* **280,** H1415–H1421
18. Stemmer, P., and Akera, T. (1988) *Biochim. Biophys. Acta* **940,** 188–196
19. Kraft, A. S., and Anderson, W. B. (1983) *Nature* **301,** 621–623
20. Henrich, C. J., and Simpson, P. C. (1988) *J. Mol. Cell. Cardiol.* **20,** 1081–1085
21. Pucéat, M., Hilal-Dandan, R., Strulovici, B., Brunton, L. L., and Heller Brown, J. H. (1994) *J. Biol. Chem.* **269,** 16938–16944
22. Clerk, A., Bogoyevitch, M. A., Andersson, M. B., and Sugden, P. H. (1994) *J. Biol. Chem.* **269,** 32848–32857
23. Johnson, J. A., and Mochly-Rosen, D. (1995) *Circ. Res.* **76,** 654–663
24. Goldberg, M., Zhang, H. L., and Steinberg, S. F. (1997) *J. Clin. Invest.* **99,** 55–61
25. Puceat, M., and Vassort, G. (1996) *Mol. Cell. Biochem.* **157,** 65–72
26. Rhee, S. G., and Bae, Y. S. (1997) *J. Biol. Chem.* **272,** 15045–15048
27. Gusovsky, F., Hollingsworth, E. B., and Daly, J. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 3003–3007
28. Ho, A. K., Ceña, V., and Klein, D. C. (1987) *Biochem. Biophys. Res. Commun.* **142,** 819–825
29. Otani, R., Otani, H., Morita, M., and Das, D. K. (1989) *Mol. Cell. Biochem.* **90,** 111–120
30. Gotoh, H., Kamiyama, A., Shibayama, R., Sawada, M., and Kashimoto, T. (1993) *Biochem. Biophys. Res. Commun.* **194,** 72–78
31. Muller-Decker, K. (1989) *Biochem. Biophys. Res. Commun.* **162,** 198–205
32. Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992) *Cell* **71,** 765–776
33. Sadoshima, J., and Izumo, S. (1993) *EMBO J.* **12,** 1681–1692
34. Jiang, T., Kuznetsov, V., Pak, E., Zhang, H., Robinson, R. B., and Steinberg, S. F. (1996) *Circ. Res.* **78,** 553–563
35. Schwartz, A., Grupp, G., Wallick, E., Grupp, I. L., and Ball, W. J., Jr. (1988) *Prog. Clin. Biol. Res.* **268B,** 321–338
36. Akera, T., and Ng, Y. C. (1991) *Life Sci.* **18,** 135–142
37. Grupp, G., DePover, A., Grupp, I. L., and Schwartz, A. (1984) *Proc. Soc. Exp. Biol. Med.* **175,** 39–43

38. Marais, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) *EMBO J.* **14,** 3136–3145
39. Morrison, D. K., and Cutler, R. E., Jr. (1997) *Curr. Opin. Cell Biol.* **9,** 174–179
40. Chang, B. Y., Chiang, M., and Cartwright, C. A. (2001) *J. Biol. Chem.* **276,** 20346–20356
41. Mochly-Rosen, D., and Gordon, A. S. (1998) *FASEB J.* **12,** 35–42
42. Tian, J., Gong, X., and Xie, Z. (2001) *Am. J. Physiol.* **281,** H1899–H1907
43. Ward, C. A., and Moffat, M. P. (1992) *J. Mol. Cell. Cardiol.* **24,** 937–948
44. Nicolás, J. M., Renard-Rooney, D. C., and Thomas, A. P. (1998) *J. Mol. Cell. Cardiol.* **30,** 2591–2604
45. Ewart, H. S., and Klip, A. (1995) *Am. J. Physiol.* **269,** C295–C311
46. Therien, A. G., and Blostein, R. (2000) *Am. J. Physiol.* **279,** C541–C566
47. Feschenko, M. S., and Sweadner, K. J. (1995) *J. Biol. Chem.* **270,** 14072–14077
48. Feschenko, M. S., and Sweadner, K. J. (1997) *J. Biol. Chem.* **272,** 17726–17733
49. Lundmark, J. L., Ramasamy, R., Vulliet, P. R., and Schaefer, S. (1999) *Am. J. Physiol.* **277,** H999–H1006
50. Efendiev, R., Bertorello, A. M., Pressley, T. A., Rousselot, M., Féraille, E., and Pedemonte, C. H. (2000) *Biochemistry* **39,** 9884–9892
51. Naruse, K., and King, G. L. (2000) *Circ. Res.* **86,** 1104–1106
52. Bueno, O. F., De Windt, L. J., Tymitz, K. M., Witt, S. A., Kimball, T. R., Levitsky, R., Hewett, T. E., Jones, S. P., Lefer, D. J., Peng, C-F., Kitsis, R. N., and Molkentin, J. C. (2000) *EMBO J.* **19,** 6341–6350