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Protein Kinase-mediated Regulation of the Na^+/H^+ Exchanger in the Rat Myocardium by Mitogen-activated Protein Kinase-dependent Pathways*

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Andrea N. Moor and Larry Fliegel‡

From the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

We examined regulation of the Na^+/H^+ exchanger isoform 1 by phosphorylation in the rat myocardium. We utilized cell extracts from adult rat hearts, adult rat extracts fractionated by fast performance liquid chromatography, and extracts from cultured neonatal cardiac myocytes. The carboxyl-terminal 178 amino acids of the Na^+/H^+ exchanger were expressed in *Escherichia coli* fused with glutathione *S*-transferase. The purified protein was used as a substrate for *in vitro* phosphorylation and in-gel kinase assays. Unfractionated extracts from neonatal myocytes or adult hearts phosphorylated the COOH-terminal domain of the antiporter. Western blot analysis revealed that mitogen-activated protein (MAP) kinase (44 and 42 kDa) and $p90^{rsk}$ (90 kDa) were present in specific fractions of cardiac extracts that phosphorylated the COOH-terminal protein. In-gel kinase assays confirmed that protein kinases of approximately 44 and 90 kDa could phosphorylate this domain. MAP kinase and $p90^{rsk}$-dependent phosphorylation of the antiporter could be demonstrated by immunoprecipitation of these kinases from extracts of neonatal cardiac myocytes. PD98059, a mitogen-activated protein kinase kinase inhibitor, decreased MAP kinase and $p90^{rsk}$ phosphorylation of the antiporter and abolished serum and endothelin 1-stimulated increases in steady-state pH_i. These results confirm the presence of MAP kinase-dependent phosphorylation in the regulation of the Na^+/H^+ exchanger in the rat myocardium and suggest an important role for $p90^{rsk}$ phosphorylation in regulation of the protein by endothelin-mediated stimulation of the antiporter.

The Na^+/H^+ exchanger isoform-1 (NHE1)[1] is a ubiquitously expressed integral membrane protein (1) with a molecular mass of about 100 kDa (2). It localizes to the plasma membrane, where it functions in the maintenance of cytosolic pH (pH_i) and intracellular volume (3). Six isoforms (NHE1–NHE6) exist, which vary in molecular weight and in sensitivity to the inhibitor amiloride (1, 4).

The exchanger functions by extruding one proton in exchange for one sodium ion when pH_i is too acidic (5). An amino-terminal membrane domain mediates transport, and a cytosolic, hydrophilic carboxyl-terminal domain of approximately 300 amino acids in length (1, 3) regulates activity. The Na^+/H^+ exchanger is the protein responsible for removing most acid equivalents in the myocardium, particularly in cases of acute acid load (6). It is suggested that Na^+/H^+ exchange is important in modulating the cardiac response to reperfusion after ischemia (7). The increases in intracellular sodium from the activity of the exchanger result in calcium overload by the Na^+/Ca^{2+} exchanger and subsequently lead to increased damage to the myocardium (7). These facts underlie the importance of understanding the regulatory mechanisms functioning to control Na^+/H^+ exchange.

Studies have shown that phosphorylation of the Na^+/H^+ exchanger only occurs in the distal region of the cytosolic domain (8). Regulation of exchanger activity by phosphorylation of the carboxyl-terminal domain (last 178 amino acids) of the protein has been directly demonstrated using specific kinases present in rabbit skeletal muscle (9) and rat vascular smooth muscle cells (10). To date, however, no studies have examined regulation of Na^+/H^+ exchanger activity by kinases isolated from the myocardium. In this report, we identified two myocardial kinases important in the regulation of the COOH-terminal domain of NHE1 MAP kinase and $p90^{rsk}$ or Rsk (a substrate for MAP kinase phosphorylation). The results implicate a MAP kinase signaling pathway in the regulation of the COOH-terminal domain of the NHE1 isoform of the Na^+/H^+ exchanger and give the first direct evidence of myocardial kinases involved in this regulation.

EXPERIMENTAL PROCEDURES

Materials—PD98059, a MEK inhibitor, was from Calbiochem-Novabiochem Corp. Plasmid pGEX-3X, glutathione-Sepharose 4B affinity column, and protein A/G-Sepharose CL-4B beads were from Amersham Pharmacia Biotech (Uppsala, Sweden). Anti-MAP kinase R2 (Erk1-CT) was from Kinetek Pharmaceuticals, Inc. (Vancouver, Canada), while Anti-ERK-1 (rabbit polyclonal), anti-p-ERK (mouse monoclonal), and anti-Rsk-1 (goat polyclonal) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-RSK ($p90^{rsk}$) (mouse monoclonal) was from Transduction Laboratories (Lexington, KY). MF-20 (mouse monoclonal) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech (Oakville, Canada), and [^{32}P]orthophosphate was obtained from ICN Radiochemicals (Irvine, CA). Collagenase type 2 was obtained from Worthington, and BCECF-AM was from Molecular Probes, Inc. (Eugene, OR).

Construction and Purification of Glutathione S-Transferase-Na^+/H^+ Exchanger Fusion Protein—The carboxyl-terminal 178-amino acid sequence of the rabbit cardiac Na^+/H^+ exchanger was expressed as de-

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‡ A Senior Scholar of the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed: Dept. of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-1848; Fax: 780-492-0886; E-mail: lfliegel@gpu.srv.ualberta.ca.

[1] The abbreviations used are: NHE1, Na^+/H^+ exchanger isoform-1; ERK, extracellular signal regulated kinase; FPLC, fast performance liquid chromatography; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; pH_i, intracellular pH; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; RIPA, radioimmune precipitation buffer; ET, endothelin.

scribed previously (9) as a fusion protein with GST (PCRA or PCR178) using the plasmid pGEX-3X (9). The *Escherichia coli* TOPP 2 strain was induced with 1 mM isopropylthio-β-D-galactoside. PCRA was purified via glutathione-Sepharose 4B affinity chromatography as described earlier (9).

Preparation and Fractionation of Cell Extracts from Adult Rat Myocardium—Extracts were prepared from ventricles of untreated adult Harlan Sprague-Dawley rats. The heart tissue was homogenized at a high setting with a Polytron homogenizer for 30 s in 2.5 (v/w) of extraction buffer containing 10 mM sodium phosphate, 60 mM β-glycerophosphate, 1 mM dithiothreitol, 15 mM EGTA, 15 mM magnesium chloride, pH 7.5, 10 mM phenylmethylsulfonyl fluoride in methanol, 1 mM benzamidine hydrochloride in ethanol, and a protease inhibitor mixture (12). The homogenate was centrifuged at 6000 × *g* for 60 min at 4 °C. The supernatant was then centrifuged at 10,000 × *g* for 60 min at 4 °C. Muscle extracts were fractionated on a Mono Q 10 column using FPLC as described previously (9). Western blot analysis was done using commercially available antibodies against MAP kinase (ERK-1) and $p90^{rsk}$.

In Vitro Phosphorylation of the Na^+/H^+ Exchanger Fusion Protein (PCRA)—The standard reaction conditions for phosphorylation of heart extract fractions contained 3.0–8.0 μg of PCRA, 8 μl of heart extract, 12.5 mM MOPS, pH 7.2, 0.5 mM EGTA, 2 mM dithiothreitol, 8.5 mM magnesium chloride, 6 μM okadaic acid, 0.24 mM sodium fluoride, 500 μM ATP, and 1 μl of 10 μCi/μl [γ-^{32}P]ATP (3000 Ci/mmol) in a final volume of 24 μl (modified according to Ref. 9). Samples were incubated at 30 °C for 90 min, and the reaction was terminated by the addition of SDS loading buffer. Samples were run on a 12% SDS gel, dried, and exposed for autoradiography. The stoichiometry of phosphorylation was determined by two methods using samples separated by SDS-PAGE. The appropriate bands were identified by autoradiography and excised, and incorporation was measured by liquid scintillation counting. The same results were obtained by quantification using a model BAS1000 phosphor imager (Fuji Photo Film Co., Ltd.) to examine radioactivity incorporated into the protein bands.

In-gel Kinase Assays—To identify specific kinases that phosphorylated PCRA, heart fractions of interest were separated by 10% SDS-PAGE in a gel containing 1 mg/ml of PCRA. In-gel kinase assays were as described earlier (9). The gel was dried for autoradiography and visualization of phosphorylation (13).

Primary Cultures of Isolated Neonatal Myocytes—Primary myocyte cultures were prepared from neonatal Harlan Sprague-Dawley rats as described previously (14). Isolated primary myocytes were plated onto glass coverslips for physiologic studies or onto Primaria™ (Falcon) culture dishes or flasks. Myocytes were maintained for 4–5 days in medium containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mg/ml bovine serum albumin, 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium nonessential amino acids, 10% minimum essential medium vitamin, 0.1 mM bromodeoxyuridine, 100 μM L-ascorbic acid, and 30 mM HEPES, pH 7.1. Cells were serum-starved overnight prior to experiments when myocytes were stimulated with serum or ET-1.

Measurement of Na^+/H^+ Exchanger Activity—Myocytes were grown on glass coverslips, and the acetoxy methyl ester of 2′-7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein was used to measure pH_i as described earlier (9, 14, 15). Excitation wavelengths were at 452 and 503 nm with emission at 524 nm. Myocytes were serum-starved before pH_i measurement, and the normal buffer used for pH measurements contained 137 mM sodium chloride, 1.6 mM magnesium sulfate, 5.4 mM potassium chloride, 1.2 mM sodium dihydrogen orthophosphate, 20 mM HEPES, 15 mM D-glucose, and 1.0 mM calcium chloride, pH 7.1. Steady-state pH_i was measured following a brief acid challenge using ammonium chloride prepulse (20 mM for 2–3 min) followed by ammonium chloride-free buffer pH 7.1 (14). After recovery of a stable resting pH_i, either serum (10%) or ET-1 (0.5 nM) was added, and changes in resting pH_i were measured for 10 min. In some experiments, the cells were either pretreated with the MEK inhibitor PD98059 (50 μM) or Me_2SO vehicle for 2 min followed by the above stimulation with either serum or ET-1.

Endogenous Phosphorylation of NHE1 of Neonatal Cardiac Myocytes—To examine phosphorylation of NHE1 *in vivo*, neonatal cardiac myocytes were incubated in a phosphate-free buffer containing 130 mM sodium chloride, 5 mM potassium chloride, 1.8 mM calcium chloride, 1.0 mM magnesium sulfate, 5.0 mM D-glucose, 20.0 mM HEPES, 2.0 mM glutamine, and 1.0 g/liter bovine serum albumin, pH 7.4, for 30 min at 37 °C (16, 17). Myocytes were then incubated with [^{32}P]orthophosphate (100 μCi/ml) for 3 h in phosphate-free buffer at 37 °C. Five minutes before the incubation was terminated, myocytes were stimulated with FBS (10%) for 5 min. Unstimulated myocytes were used as controls. In some experiments, myocytes were treated with PD98059 prior to immunoprecipitation as described below. Cells were then washed with cold phosphate-free buffer, and this was replaced with a cold buffer (RIPA) containing 150 mM sodium chloride, 80 mM sodium fluoride, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM EGTA, 25 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate (17) and placed on dry ice for 5–10 min. Myocytes were thawed on ice, scraped into centrifuge tubes, sonicated for 10 s, and centrifuged at 35,000 rpm at 4 °C for 1 h. The supernatant was removed, and RIPA buffer containing 1% (v/v) Nonidet P-40 (Nonidet P-40), 0.5% (w/v) deoxycholate, and 0.1% (w/v) SDS was added. The solution was centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant was frozen in liquid nitrogen and kept at −80 °C overnight.

Immunoprecipitations were with affinity-purified NHE1 antibodies against the Na^+/H^+ exchanger (2). To reduce nonspecific binding to Sepharose, radiolabeled myocyte extracts (1 ml) were treated with protein A-Sepharose CL-4B beads for 30 min. These samples were centrifuged for 1–2 min at 7000 rpm at 4 °C, and the supernatant was retained. Sepharose beads used for immunoprecipitation were also pretreated to reduce nonspecific binding. Unlabeled myocyte extract was combined with protein A-Sepharose CL-4B beads for 2 h at 4 °C, and the beads were washed with RIPA. For immunoprecipitations, 30 μl of anti-NHE1 antibody (2) was added to the pretreated supernatant, and this was agitated for 2 h at 4 °C. The antibody-antigen complex was then added to pretreated beads and agitated for 1 h at 4 °C. The beads were washed extensively with RIPA buffer and then solubilized in SDS-PAGE gel sample buffer. The immunoprecipitate was boiled and run on a SDS-PAGE. Finally, the gel was dried for autoradiographic analysis. In some experiments, immunoprecipitates were transferred to Nitrocellulose and probed with antibody against the antiporter to confirm their identity. Immunoprecipitations were repeated three times in experiments comparing FBS-stimulated and -unstimulated cells, and they were repeated two times in experiments comparing PD98059-treated and -untreated cells. In the experiments with PD98059, the amount of immunoprecipitate was quantified by Western blotting and densitometric analysis to ensure equivalent amounts of protein were compared.

Isolation of Cell Lysates Containing Activated MAP Kinase and $p90^{rsk}$ from Neonatal Cardiac Myocytes—To characterize *in vivo* stimulated kinases, myocytes were serum-starved overnight. To mimic conditions of the experiments measuring pH_i, myocytes were subjected to a brief ammonium chloride prepulse as described above. Myocytes were then exposed to either 10% FBS or 0.5 nM ET-1 for 0–5 min at 37 °C. Unstimulated myocytes were used as controls. In some experiments, the cells were either pretreated with the MEK inhibitor PD98059 (50 μM) or Me_2SO vehicle for 30 min followed by stimulation with either serum or ET-1. The myocytes were washed with ice-cold phosphate-buffered saline and an extraction buffer containing 50 mM tetrasodium pyrophosphate, 50 mM sodium fluoride, 50 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 0.1 mM sodium orthovanadate, 0.01% Triton X-100, 10 mM HEPES, pH 7.4, and a mixture of protease inhibitors. The cells were frozen on dry ice for 5 min, allowed to thaw on ice for 15 min, scraped, and transferred into microcentrifuge tubes. The myocyte extracts were sonicated for 10 s on ice and then centrifuged at 10,000 rpm for 30 min at 4 °C. Extracts were analyzed by Western blot analysis using antibodies against MAP kinase (ERK-1), phosphorylated MAP kinase (p-ERK), cardiac myosin heavy chain (MF-20), and $p90^{rsk}$ and for their ability to phosphorylate PCRA fusion protein using the assays described earlier. For in-gel kinase assays of stimulated and unstimulated neonatal cardiac myocytes, cell lysates were made as described above. Equal amounts of protein were then used for the in-gel kinase assays. We confirmed that equal amounts of protein were applied to the gels by Coomassie Blue staining of identically run gels or by Western blot analysis of identically run samples with an antibody against either MAP kinase or $p90^{rsk}$. Protein concentrations were measured using the Bio-Rad DC protein assay.

Immunoprecipitation of MAP Kinase and $p90^{rsk}$ from Myocyte Extracts—For immunoprecipitation, anti-ERK-1 and anti-Rsk-1 antibodies were used. Myocyte lysates (500 μl) were pretreated by incubating with protein A-Sepharose Cl-4B beads (in experiments with anti-ERK-1 antibodies) or protein G-Sepharose beads (in experiments with anti-Rsk-1) for 30 min. The samples were centrifuged for 1–2 min at 7000 rpm at 4 °C to remove nonspecifically adsorbed proteins bound to the resins. In addition, Sepharose beads used for immunoprecipitation were pretreated to reduce nonspecific binding. They were incubated with unlabeled myocyte extract for 2 h at 4 °C and washed with extraction

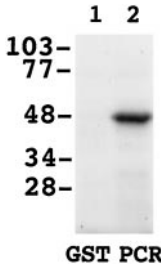

FIG. 1. **Phosphorylation of Na^+/H^+ exchanger fusion protein by unfractionated cardiac muscle extracts.** Samples were incubated in buffer containing [γ-^{32}P]ATP, unfractionated cell extracts, and fusion protein. The autoradiogram (*lane 1*) shows that purified GST protein is not phosphorylated by cardiac muscle cell extracts, while PCRA-GST (*lane 2*) shows phosphorylation.

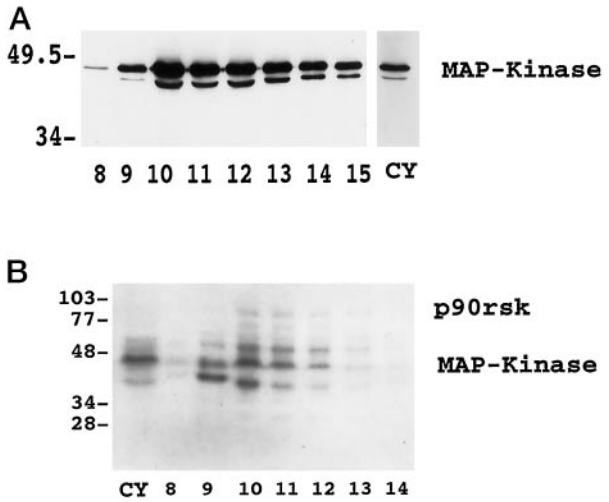

FIG. 2. **Identification of MAP kinase in adult rat cardiac cell extracts.** Western blot analysis was performed with anti-MAP kinase R2 (rabbit polyclonal, 1:4000) antibodies against fractionated cell extracts (*lanes 8–15*) and unfractionated extract (*lane CY*). *A*, Western blot of fractions 8–15 and unfractionated extracts with anti-MAP kinase antibody (immunoreactive with 44- and 42-kDa isoforms). *B*, in-gel kinase assay using PCRA-GST as a substrate (1 mg/ml); unfractionated cardiac extracts and fractionated cardiac cell extracts (*lanes 8–14*) are shown.

buffer. For immunoprecipitation of protein kinases, 10 μl of 200 μg/ml stock anti-ERK-1 antibody or 10 μl of 200 μg/ml stock anti-Rsk-1 antibody was added to pretreated myocyte lysate, and this mixture was rotated for 2 h at 4 °C. The antibody-antigen complex was then added to pretreated beads and rotated for 1 h at 4 °C. The beads were washed extensively in extraction buffer and then solubilized in SDS-PAGE gel sample buffer. The immunoprecipitate was analyzed by in-gel kinase assay.

Statistics—Analysis of results was by a Mann-Whitney *U* test and/or Student's unpaired *t* test on direct numerical values obtained (18). Values presented are mean ± S.E. of the controls with the number of experiments indicated; p values < 0.05 were considered statistically significant.

RESULTS

We produced the carboxyl-terminal 178 amino acids as a fusion protein with GST (46.9 kDa). The identity of the purified protein was confirmed using an antibody generated against the carboxyl-terminal region of the Na^+/H^+ exchanger (11) (not shown). We used this protein to examine regulation of the Na^+/H^+ exchanger by phosphorylation in the mammalian myocardium. Initially, we isolated a cell extract from the adult rat myocardium, and this extract was fractionated on a Mono Q column. Both unfractionated and fractionated extract were tested for their ability to phosphorylate PCRA-GST. Fig. 1 shows that unfractionated extract does not phosphorylate purified GST protein (*lane 1*) but does phosphorylate purified PCRA-GST fusion protein that contains the terminal 178 amino acids of the Na^+/H^+ exchanger (46–47 kDa) (*lane 2*). The stoichiometry of phosphorylation was 1–2 mol of P_i/mol of PCRA-GST. A time course of phosphorylation showed that the fusion protein was fully phosphorylated (not shown).

To examine which kinases might be involved in the phosphorylation of the fusion protein, we analyzed the extracts by Western blot analysis using commercially available antibodies against MAP kinase (ERK-1) (Fig. 2*A*). Unfractionated extract (*CY*) contained both MAP kinase (44 and 42 kDa) isoforms as well as a small amount of the kinase p90^{rsk} (90 kDa) when probed with an antibody to p90^{rsk} (not shown). Fractions 10–12 were enriched in MAP kinase relative to other fractions, and these same fractions contained small amounts of p90^{rsk} protein (not shown).

To identify potential protein kinases involved in regulation of the Na^+/H^+ exchanger, we used in-gel kinase assays. In-gel kinase assays with GST alone as a substrate showed no discrete phosphorylation patterns and no evidence that kinases were phosphorylating GST to any significant extent (not shown). However, assays with GST-PCRA as a substrate showed that a number of kinases of discreet molecular sizes could phosphorylate this fusion protein (Fig. 2*B*). In unfractionated samples (*CY*) the major kinase activity was of approximately 44 kDa. In addition, there was a minor band of approximately 38 kDa. Fractions 9–12 contained bands of kinase activity corresponding to molecular masses of approximately 44, 90, 60, and 38 kDa (Fig. 2*B*). A weak band was also noticed of approximately 75 kDa. The finding of kinases of about 44 and 90 kDa was consistent with the size and activity of MAP kinase and p90^{rsk}. We did not find evidence for significant activity of protein kinases larger than 90 kDa.

We next characterized steady state physiological pH_i regulation by the Na^+/H^+ exchanger in isolated neonatal myocytes stimulated with either ET-1 or FBS. Steady state monitoring of pH_i allowed us to observe a continuous time course of events. Myocytes were serum-starved overnight. Fig. 3*A* shows typical effects of the addition of ET-1 or FBS on steady-state pH_i of neonatal cardiac myocytes, and Fig. 3*B* summarizes the results. Both ET-1 and FBS addition raised the steady-state pH_i to more alkaline values compared with unstimulated cardiac myocytes (control). The MEK inhibitor PD98059 abolished the effects of ET-1 and FBS addition, showing that the above effects were due to activation of the Na^+/H^+ exchanger via the MAP kinase signal transduction pathway. A summary of a series of experiments is shown in Fig. 3*B*.

We then examined *in vivo* phosphorylation of NHE1 in neonatal cardiac myocytes. Affinity-purified anti-Na^+/H^+ exchanger antibody was used to immunoprecipitate the exchanger from equal amounts of isolated myocytes. Fig. 4*B* shows typical results and illustrates that FBS stimulation increased the *in vivo* phosphorylation state of the NHE1 compared with unstimulated myocytes. Scanning densitometry estimated a 2–3-fold increase in the phosphorylation level in stimulated cells. Fig. 4*A* shows a typical Western blot of the immunoprecipitate probed with anti-NHE1 antibody. The result confirms that we were immunoprecipitating Na^+/H^+ exchanger protein. Fig. 4*C* shows that PD98059 dramatically reduced the phosphorylation of the antiporter that was induced by serum. Scanning densitometry estimated that there was approximately a 5-fold decrease in the level of phosphorylation.

To examine mechanisms behind the physiological effects, we obtained cell extracts from neonatal cardiac myocytes that were unstimulated (control) (Fig. 5*A*, *lane 1*), ET-1-stimulated (*lane 2*), and FBS-stimulated (*lane 3*). All of the extracts had

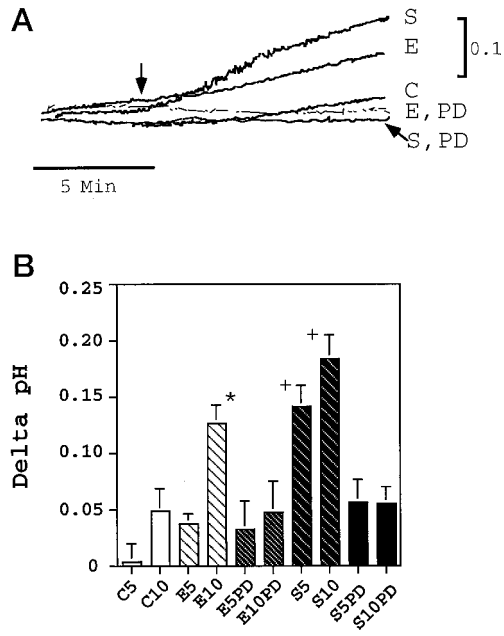

FIG. 3. **Effect of ET-1 and FBS stimulation on steady state pH_i of neonatal cardiac myocytes.** Myocytes were grown on coverslips and maintained as described under "Experimental Procedures." Cells were serum-starved overnight prior to stimulation. After the addition of either ET-1 or FBS, the changes from resting pH_i were measured for 10 min, and results were summarized at 5- and 10-min intervals. *A*, examples of typical effects of ET-1 (*E*) and FBS (*S*) addition on pH_i. *Trace C*, unstimulated or control myocytes. Traces *E*, *PD* (*thin line*) and *S*, *PD* show the effect of pretreating the myocytes with PD98059 (50 μM) prior to stimulation with either ET-1 (0.5 nM) or FBS (10%). *B*, bar graph summarizing the effects of the addition of ET-1 or FBS (with or without PD98059) on resting pH_i. Results are the mean ± S.E. of at least five experiments. An *asterisk* indicates significant difference from control values at $p < 0.05$; +, $p < 0.01$. *Numbers* (5 or 10) indicate the time after the addition of serum or ET-1.

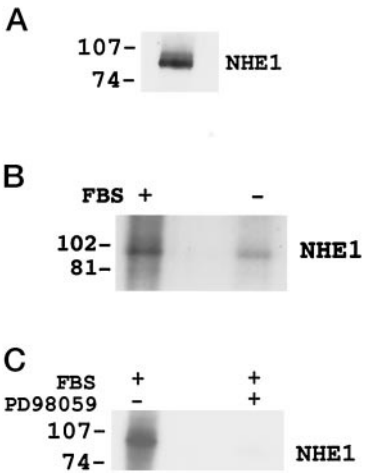

FIG. 4. ***In vivo*** **phosphorylation of NHE1 from neonatal cardiac myocytes.** Neonatal cardiac myocytes were labeled with ^{32}P, and the endogenous Na^+/H^+ exchanger protein was immunoprecipitated using affinity-purified antibodies. *A*, Western blot of the immunoprecipitate with anti-exchanger antibodies. *B*, autoradiogram illustrating phosphorylation state of the endogenous protein (approximately 100 kDa) from FBS-stimulated (+) (5 min) and unstimulated (−) neonatal cardiac myocytes. *C*, autoradiogram illustrating phosphorylation state of the endogenous protein from FBS-stimulated (5 min) neonatal cardiac myocytes either treated (+) or untreated (−) with 50 μM PD98059 as described under "Experimental Procedures."

the ability to phosphorylate PCRA-GST (46–47 kDa) in *in vitro* phosphorylation assays. However, extracts from ET-1 and FBS-stimulated cardiac myocytes showed a 2-fold increase in

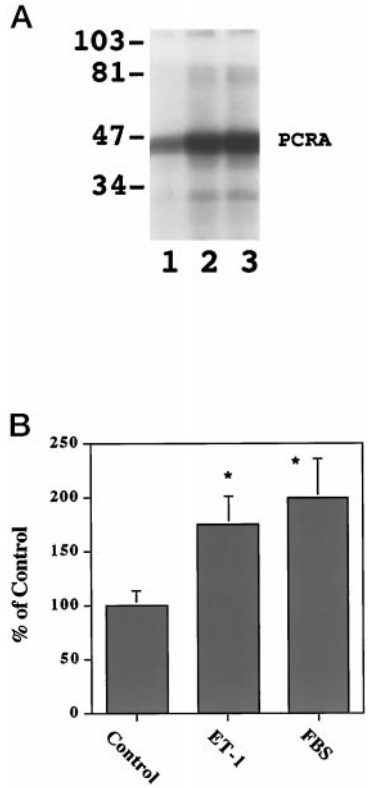

FIG. 5. **Regulation of protein kinase-mediated phosphorylation of the Na^+/H^+ exchanger by cell extracts of neonatal myocytes.** Cell extracts from isolated, neonatal cardiac myocytes were either unstimulated or stimulated with ET-1 (0.5 nM) or FBS (10%) for 5 min. Samples were incubated in a buffer containing [γ-^{32}P]ATP, neonatal myocyte cell extracts, and fusion protein as described under "Experimental Procedures." *A*, autoradiography of stimulated and unstimulated cell extracts. *Lane 1*, basal levels of phosphorylation of the Na^+/H^+ exchanger fusion protein (46 kDa). *Lane 2*, phosphorylation by extracts from ET-1-stimulated myocytes. *Lane 3*, phosphorylation of PCRA-GST by extracts from FBS-stimulated myocytes. *B*, bar graph summarizing the results of *A*. Results are mean ± S.E. of three experiments. *, $p < 0.05$.

phosphorylation of PCRA-GST ($p < 0.05$) compared with controls (Fig. 5*B*).

To confirm that myocyte extracts contained equal amounts of protein and kinases, SDS-PAGE and Western blot analysis routinely analyzed cell lysates. Antibodies against MAP kinase (ERK-1) and $p90^{rsk}$ showed that all of the extracts contained equal amounts of these two kinases (Fig. 6*A*) (*lane 1*, control extract; *lane 2*, extract from ET-1-stimulated myocytes; *lane 3*, extract from FBS-stimulated myocytes). Equal amounts of protein were obtained from the different cells (0.8–1.0 μg/μl).

To confirm that the 44- and 90-kDa bands were MAP kinase and $p90^{rsk}$, respectively, we used immunoprecipitation with anti-MAP kinase (44 kDa) and anti-$p90^{rsk}$ (90 kDa) antibodies. The proteins were removed from neonatal cardiac cell extracts with antibodies as described under "Experimental Procedures." The immunoprecipitates were tested for their ability to phosphorylate the carboxyl terminus of the Na^+/H^+ exchanger in an in-gel kinase assays (Fig. 6*B*). *Lanes 1* and *3* show phosphorylation by the 42- and 44-kDa isoforms of MAP kinase and by $p90^{rsk}$ (Fig. 6*B*). Anti-MAP kinase antibody immunoprecipitated a band corresponding in size to the major 44-kDa kinase we found in cell extracts and was equivalent in size to that of MAP kinase (Fig. 6*B*, *lane 2*). Anti-$p90^{rsk}$ antibody immunoprecipitated a band that corresponded in size to $p90^{rsk}$ kinase and to the 90-kDa band we observed in whole cell and tissue extracts (Fig. 6*B*, *lane 4*). A control experiment in which no

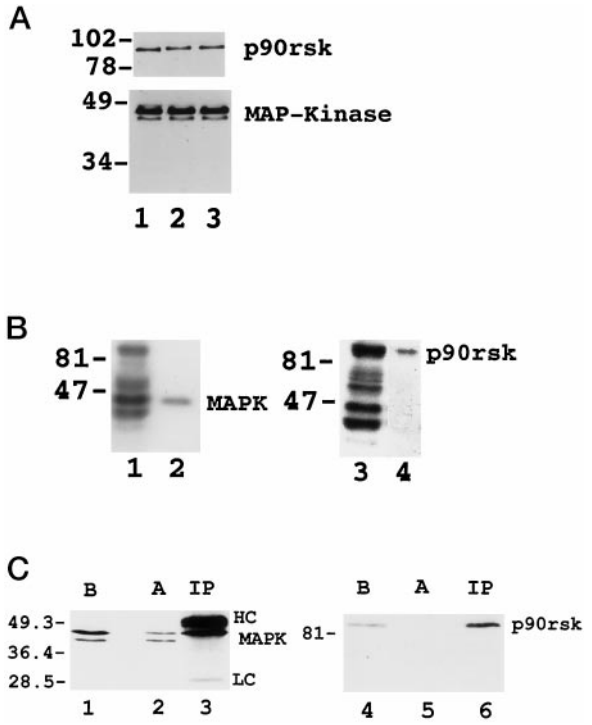

FIG. 6. **Characterization of cell extracts of neonatal cardiac myocytes.** Cell extracts were prepared from control (*lane 1*), ET-1-treated (*lane 2*), and FBS-treated (*lane 3*) neonatal cardiac myocytes. Western blot analysis was performed using anti-MAP kinase R2 (rabbit polyclonal, 1:4000) and anti-$p90^{rsk}$ (mouse monoclonal, 1:100) antibodies. *A*, extracts immunostained with anti-$p90^{rsk}$ (90 kDa) antibody and with anti-MAP kinase antibody. *B*, autoradiogram showing in-gel kinase assay using Na^+/H^+ exchanger fusion protein (PCRA) as a substrate (1 mg/ml). *Lanes 1* and *3* show phosphorylation of PCRA-GST by kinases from neonatal myocyte cell extracts before immunoprecipitation of MAP kinase and $p90^{rsk}$. *Lane 2* shows the specific phosphorylation observed using a sample immunoprecipitated with anti-MAP kinase antibody. *Lane 4* shows the specific phosphorylation observed using a sample immunoprecipitated with anti-$p90^{rsk}$ antibodies. *C*, Western blot of immunoprecipitation of MAP kinase and $p90^{rsk}$. *Lanes 1–3*, anti-MAP-kinase antibody. *Lanes 4–6*, $p90^{rsk}$ antibody. Lane *1* and *4*, cell extract B, before immunoprecipitation; *lanes 2* and *5*, cell extract A, after immunoprecipitation. *IP*, immunoprecipitate of MAP kinase antibody (*lane 3*) and $p90^{rsk}$ antibody (*lane 6*).

primary antibody was added did not show either a 42–44-kDa band or a 90-kDa band (not shown). Fig. 6*C* confirms that we immunoprecipitated the appropriate protein kinases. *Lane 1* shows MAP kinase of a control cell extract, while *lane 2* shows an extract in which much of the MAP kinase was removed by immunoprecipitation. *Lane 3* shows immunoprecipitate of MAP kinase protein. The heavy and light chains of the IgG were also evident, but in control experiments they did not account for the MAP kinase band (not shown). *Lane 4* shows $p90^{rsk}$ of a control cell extract, and *lane 5* shows the extract after immunodepletion with anti-$p90^{rsk}$ antibody. Lane 6 shows that the immunoprecipitate contains $p90^{rsk}$.

We performed time course experiments on the phosphorylation levels of MAP kinase (Fig. 7*A*) and $p90^{rsk}$ (Fig. 8, *A* and *B*) and then used in-gel kinase assays to examine if MAP kinase (Fig. 7*B*) and $p90^{rsk}$ (Fig. 9, *A* and *B*) respond to the same physiological stimuli that affected pH_i regulation. Fig. 7*A* shows phosphorylation levels of MAP kinase in total cell extracts detected with antibodies to the phosphorylated form of MAP kinase (p-ERK). Control cells were unstimulated, while other cells were FBS-stimulated for 30 s to 5 min. The same extracts were probed with anti-MF-20 (antibody to cardiac myosin heavy chain) to show equal amounts of protein loaded in each lane. Clearly, MAP kinase is highly phosphorylated

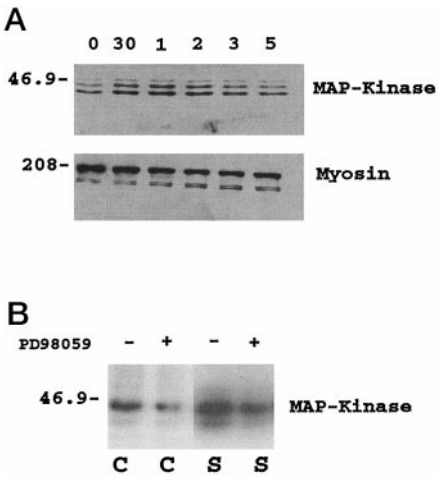

FIG. 7. **Time course of MAP kinase phosphorylation.** *A*, immunoblot of phosphorylation levels of MAP kinase in total cell extracts detected with antibodies to the phosphorylated form of MAP kinase (p-ERK, mouse monoclonal, 1:1500). Control cells were unstimulated, while other cells were FBS-stimulated for 30 s to 5 min. The same extracts were probed with anti-MF-20 (antibody to cardiac myosin heavy chain, mouse monoclonal, 1:500) to show equal amounts of protein loaded in each lane. *B*, autoradiogram of in-gel kinase assays showing phosphorylation of PCRA by neonatal cardiac myocyte extracts unstimulated (*C*) or stimulated by FBS (10%) for 1 min (*S*). Na^+/H^+ exchanger fusion protein (PCRA) was used as a substrate (1 mg/ml) for in-gel kinase assays. A 30-min pretreatment with PD98059 (50 μM) was used to inhibit MEK.

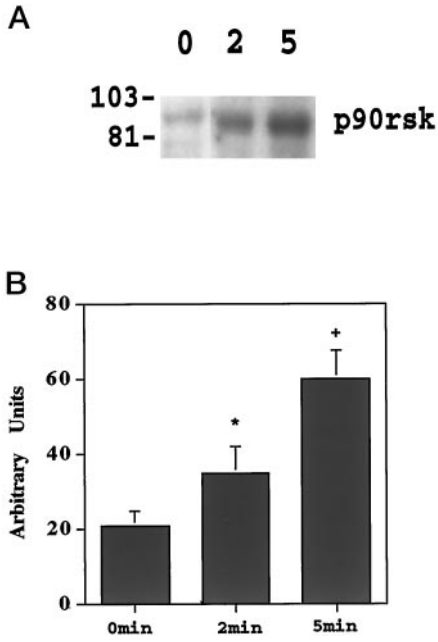

FIG. 8. **In-gel kinase assay of time course of phosphorylation of PCRA by $p90^{rsk}$.** *A*, autoradiogram showing phosphorylation of PCRA by extracts from neonatal cardiac myocytes that were stimulated for 0, 2, and 5 min with ET-1. *B*, bar graph summarizing the level of phosphorylation by $p90^{rsk}$ in ET-1-stimulated neonatal myocytes. Results are mean ± S.E. of three experiments. *, significant difference from the 5-min time point ($p < 0.05$). +, significant difference from the 0-min time point ($p < 0.01$).

within 30 s of stimulation, and this increases for up to 1 min in time. This is followed by a decrease in the level of phosphorylation of MAP kinase. Fig. 7*B* shows an in-gel kinase assay showing phosphorylation of PCRA by control myocardial cell extracts (*C*) and cell extracts from 1-min, FBS-stimulated myocytes (*S*) with and without the MEK inhibitor PD98059. The level of phosphorylation of PCRA-GST by MAP kinase from

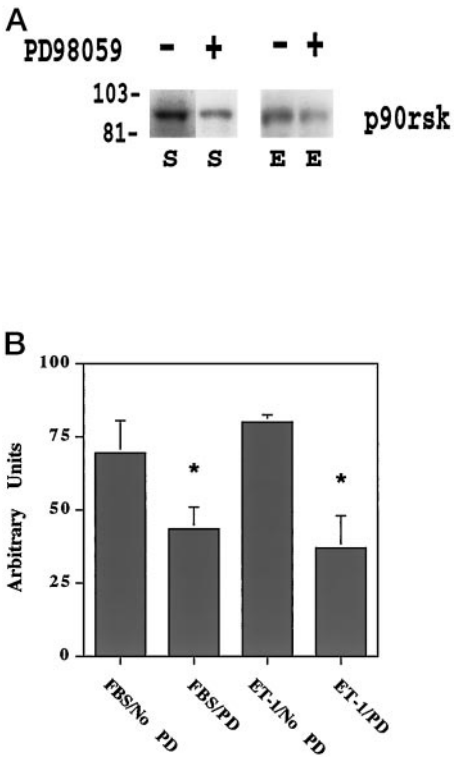

FIG. 9. **In-gel kinase assay of effect of inhibition of MEK by PD98059 on $p90^{rsk}$-mediated phosphorylation of the Na^+/H^+ exchanger.** The effect of PD98059 was examined on phosphorylation by $p90^{rsk}$. Where indicated, PD98059 (50 μM) was administered for 30 min prior to stimulation of neonatal myocytes with either ET-1 (0.5 nM for 5 min) (*lanes 2* and *4*) or FBS (10% for 5 min) (*lanes 1* and *3*). *A*, autoradiogram of typical results of $p90^{rsk}$ phosphorylation. *Lanes 1* and *2*, in the absence of PD98059; *lanes 3* and *4*, in the presence of PD98059, as indicated. *B*, bar graph summarizing the level of phosphorylation by $p90^{rsk}$ in FBS- and ET-1-stimulated neonatal myocytes with and without PD98059. Results are mean ± S.E. of three or more experiments. *, $p < 0.05$.

FBS-stimulated myocyte extracts was much greater than unstimulated (control) myocyte extracts (*lane 1 versus lane 3*). PD98059 reduced the levels of MAP kinase phosphorylation of PCRA from both control myocyte extracts and FBS-stimulated myocyte extracts (*lanes 2* and *4*).

In a second time course study, we examined the effects of 0, 2, and 5 min of ET-1 stimulation on phosphorylation of PCRA by $p90^{rsk}$ (Fig. 8*A*). The level of phosphorylation of PCRA by $p90^{rsk}$ increased significantly between the 2- and 5-min values ($p < 0.05$) (Fig. 8*B*). Thus, it appears that $p90^{rsk}$ is rapidly activated and phosphorylates the Na^+/H^+ exchanger after only 2 min of ET-1 stimulation. The activity increased even further with 5 min of stimulation.

To further characterize phosphorylation of the Na^+/H^+ exchanger by $p90^{rsk}$, we again used the MEK inhibitor PD98059. Prior to stimulation with either ET-1 or FBS, isolated cardiac myocytes were subjected to 30 min of PD98059 treatment to inhibit MEK (as above). They were then treated with ET-1 or FBS. Fig. 9*A* is an in-gel kinase assay showing non-PD98059-treated myocyte extracts from myocytes stimulated with FBS (*lanes 1* and *2*) or ET-1 (*lanes 3* and *4*). PD98059 treatment reduced $p90^{rsk}$ phosphorylation of PCRA-GST in extracts from serum- and ET-1-stimulated myocytes ($p < 0.05$) (Fig. 9). As in previous experiments, extracts from unstimulated myocytes showed only small amounts of phosphorylation, and this level was reduced significantly ($p < 0.05$) with PD98059 treatment (not shown).

DISCUSSION

The Na^+/H^+ exchanger is an essential component of pH regulation in the mammalian myocardium. Regulation of the Na^+/H^+ exchanger has still not been well characterized at the molecular level, although it is clear that phosphorylation of the distal end of the cytoplasmic domain is important in activity in some cell types (8). We characterized extracts of intact hearts to determine which kinases could be involved in regulation of the antiporter in the myocardium. We used the carboxyl-terminal 178 amino acids of the Na^+/H^+ exchanger as a substrate for protein kinases, since phosphorylation of the antiporter has been shown to be restricted to this region (6). We separated myocardial extracts into fractions with different abilities to phosphorylate the antiporter. An early peak contained MAP kinase and $p90^{rsk}$ (Fig. 2*A*). A later peak did not contain significant amounts of these kinases according to Western blot analysis (not shown). It was clear that MAP kinase- and $p90^{rsk}$-containing fractions were not the only ones capable of phosphorylating the antiporter. We characterized these fractions further using in-gel kinase assays. We found several different protein kinases that could phosphorylate the carboxyl terminus of the antiporter. In unfractionated cell lysate, the major kinase was 44 kDa (Fig. 2*B*). This was also a major band present in fractions 9–12. In addition, a 90-kDa band was present and some other kinases of varied sizes. We confirmed that the 44-kDa band and the 90-kDa band were due to MAP kinase and $p90^{rsk}$, respectively, by immunoprecipitation of these proteins with their respective antibodies (Fig. 6*B*). It was interesting to note that both in the intact heart and in extracts from cardiac myocytes, several other protein kinases could phosphorylate the Na^+/H^+ exchanger COOH terminus. Two of these were 60 and 38 kDa and were present in fractions 9–13 of the intact heart extracts. The identities of the 60- and 38-kDa kinases are not known at this time.

Immunodetection with $p90^{rsk}$ antibodies was not nearly as strong as with anti-MAP kinase antibody. This was possibly due to low levels of kinase present in this tissue or due to an antibody of lower affinity. MAP kinase and $p90^{rsk}$ are known to exist together in a complex (19). Because we had identified $p90^{rsk}$ and MAP kinase as potential protein kinases that phosphorylate the antiporter and because we have previously shown an involvement of MAP kinase-dependent pathways in other tissues (9), we examined the role of MAP kinase-dependent pathways in the myocardium in more detail.

Endothelin has been shown earlier to augment activity of the Na^+/H^+ exchanger (21). Both serum and ET-1 elevated steady state pH_i in isolated neonatal cardiac cells (Fig. 3). The MEK inhibitor PD98059 prevented the increases, suggesting that MAP kinase dependent pathways are involved. The increases in pH_i occurred even up to 10 min after administration of the stimulation. We used an intermediate time of 5 min to examine the effect of this stimulation on protein kinase activity directed toward the Na^+/H^+ exchanger. The results showed that protein kinases were activated significantly with increased activity toward the carboxyl region of the antiporter (Fig. 5). It was also clear that this type of physiological stimulation can result in increased phosphorylation of the Na^+/H^+ exchanger in cardiac cells *in vivo*. Stimulation of isolated neonatal cardiac myocytes with serum resulted in increased phosphorylation of the endogenous Na^+/H^+ exchanger in intact cells, and this effect was blocked by PD98059. Clearly, protein kinase-mediated phosphorylation of the antiporter occurs in the intact myocardium. It was most interesting that PD98059 blocks the stimulatory effect of serum on activity of the antiporter (Fig. 3) and also blocks the stimulatory effects of serum on phosphorylation of the protein (Fig. 4*C*). This suggested that MAP kinase-depend-

ent effects on phosphorylation of the antiporter *in vivo* correlated with effects on activity of the antiporter in intact cells.

We next examined which specific kinases were involved in phosphorylation that was stimulated by serum or ET-1. Stimulation with serum for 1 min increased the level of MAP kinase activity directed toward the Na^+/H^+ exchanger (Fig. 7), while stimulation with ET-1 for 2 min increased the level of $p90^{rsk}$ activity directed toward the Na^+/H^+ exchanger (Fig. 8). The MEK inhibitor PD98059 (Figs. 7 and 9) blocked this effect and also greatly reduced the basal level of phosphorylation by MAP kinase (Fig. 7) and $p90^{rsk}$ (not shown) toward the antiporter, suggesting that even in serum-deprived myocytes, a basal level of $p90^{rsk}$ and MAP kinase are involved in regulation of the antiporter.

The activity of $p90^{rsk}$ is dependent on activation by MAP kinase (22). Stimulation by serum and ET-1 probably lead to elevated MAP kinase activity that activates $p90^{rsk}$ (22). We found that MAP kinase is rapidly activated, and then its activity rapidly declines. The time course of activation of $p90^{rsk}$ was more consistent with the physiological effects we observed on activity (Fig. 3). However, it is possible that MAP kinase plays a direct role in early activation of the antiporter. The importance of the MAP kinase cascade in regulation of the antiporter has been demonstrated earlier (9, 23); however, the exact role of MAP kinase itself in regulation of the antiporter has been controversial. Earlier (9) we found an excellent time course of activation of MAP kinase activity toward the Na^+/H^+ exchanger and stimulation of Na^+/H^+ exchanger activity in smooth muscle cells. Similar to our earlier result (9), it was clear that MAP kinase could phosphorylate the antiporter directly in in-gel kinase assays. We also previously found that purified MAP kinase could phosphorylate the antiporter with a stoichiometry of 1 mol of phosphate/mol of protein. However, others were unable to demonstrate direct phosphorylation of the Na^+/H^+ exchanger using immunoprecipitated protein (23). It is known that under some conditions calmodulin (24), calcineurin homologous protein (25), and HSP70 (26) can all associate with the carboxyl terminus of the antiporter. It is possible that any of these proteins might obscure phosphorylation sites of immunoprecipitated protein. We have found that MAP kinase from three types of contractile cells (smooth muscle, skeletal (9) and cardiac cells) can directly phosphorylate the COOH terminus of the antiporter. In the present study, the results clearly showed that MAP kinase from cardiac cell extracts and from heart homogenates could phosphorylate the carboxyl terminus of the Na^+/H^+ exchanger. In in-gel kinase assays of cardiac myocytes, MAP kinase activity was the most prominent of all apparent protein kinases. The exact role and regulation of this direct phosphorylation by MAP kinase still needs further definition and may vary between cell types.

In the case of $p90^{rsk}$, a regulatory role of direct phosphorylation of the Na^+/H^+ exchanger is more apparent. In the present study, we showed that there was an excellent correlation between activation of $p90^{rsk}$ activity toward the antiporter and increased activity of the Na^+/H^+ exchanger protein *in vivo*. This was the only protein kinase in the cell extracts that showed this clear correlation with antiporter activity. $p90^{rsk}$ is clearly a strong candidate for the protein kinase mediating the effects of ET-1 on the myocardial Na^+/H^+ exchanger. Supporting this suggestion is the observation (21) that blocking G-protein-mediated effects of endothelin does not block stimulation of the Na^+/H^+ exchanger. It was previously shown that $p90^{rsk}$ is important in regulation of the Na^+/H^+ exchanger in rat vascular smooth muscle (10). In addition, phosphorylation of the Na^+/H^+ exchanger by $p90^{rsk}$ was elevated in smooth muscle cells from spontaneously hypertensive rats, which may explain the increased activity of the antiporter seen in hypertensive patients (8). Our results support a direct regulatory role of $p90^{rsk}$ on the Na^+/H^+ exchanger in the cardiac myocyte. They are the first demonstration of phosphorylation of the Na^+/H^+ exchanger in the myocardium and of the particular kinases and receptors responsive to stimulation in the myocardium. Our results differ from those reported earlier (27) in that they again show a direct phosphorylation by MAP kinase, in support of our earlier findings (9). It was curious that the phosphorylation level of MAP kinase declined rapidly, while the activity of $p90^{rsk}$ continued to increase for up to 5 min. However, it is known that MAP kinase and $p90^{rsk}$ form a complex together (19) so that a small subfraction of activated MAP kinase could activate $p90^{rsk}$. In addition, evidence has shown the $p90^{rsk}$ undergoes autophosphorylation coincident with kinase activation (29). Thus, it is possible that the further activation of $p90^{rsk}$ is the result of autophosphorylation by activated kinase.

Our results do not exclude the possibility that other protein kinases may be involved in regulation of the Na^+/H^+ exchanger with different kinds of physiological stimuli. Several consensus sequences exist in the antiporter carboxyl terminus, and more than 1 mol of phosphate is incorporated upon mitogenic stimulation of some cell types (8). A recent report (30) suggests that $p160^{ROCK}$ mediates phosphorylation of the antiporter by lysophosphatidic acid, Gα13, and RhoA. The kinase is involved in actin stress fiber formation (30). A preliminary report suggests that protein kinase D may act as a negative regulator of the antiporter (28). We found no evidence for any significant activity of protein kinases of larger apparent size than $p90^{rsk}$. However, this does not exclude a role for either of these kinases in the myocardium. It is possible that the activity of other kinases is below the detection level of this assay. It is also possible that $p160^{ROCK}$ and protein kinase D may not function well in in-gel kinase assays that require denaturation and renaturation of the kinases. In addition, in the case of protein kinase D, there is no evidence for direct phosphorylation of the exchanger. Its action may be through an intermediate protein such as calcineurin homologous protein (25). $P160^{ROCK}$ was shown to mediate direct phosphorylation of the antiporter (30) and has been shown to be present in the myocardium (20). However, it seems unlikely that it plays a role in the MAP kinase pathway stimulated by ET-1 and is more likely involved in control of the cytoskeleton by RhoA. Further studies are necessary to examine if this kinase has a role to play in the myocardium.

In summary, our results clearly show that MAP kinase-dependent pathways are important mediators of activation of the antiporter in the myocardium. The present study supports the idea that $p90^{rsk}$ is an important kinase that may be responsible for mediating the effects of ET-1 stimulation on activity of the Na^+/H^+ exchanger. MAP kinase was shown to phosphorylate the Na^+/H^+ exchanger, but its role in regulation is still unclear. We found that other protein kinases of apparent molecular mass less than 80 kDa could also phosphorylate the antiporter. Further experiments are necessary to determine their identity and possible regulatory roles in the myocardium.

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