

## Activation of Na<sup>+</sup>/H<sup>+</sup> Exchanger-directed Protein Kinases in the Ischemic and Ischemic-reperfused Rat Myocardium\*

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**The activity of the Na<sup>+</sup>/H<sup>+</sup> exchanger has been implicated as an important contributing factor in damage to the myocardium that occurs during ischemia and reperfusion. We examined regulation of the protein in ischemic and reperfused isolated hearts and isolated ventricular myocytes. In isolated myocytes, extracellular signal-regulated kinases were important in regulating activity of the exchanger after recovery from ischemia. Ischemia followed by reperfusion caused a strong inhibitory effect on NHE1 activity that abated with continued reperfusion. Four major protein kinases of size 90, 55, 44, and 40 kDa phosphorylated the Na<sup>+</sup>/H<sup>+</sup> exchanger. The Na<sup>+</sup>/H<sup>+</sup> exchanger-directed kinases demonstrated dramatic increases in activity of 2–10-fold that was induced by 3 different models of ischemia and reperfusion in intact hearts and isolated myocytes. p90<sup>rsk</sup> was identified as the 90-kDa protein kinase activated by ischemia and reperfusion while ERK1/2 was identified as accounting for some of the 44-kDa protein kinase phosphorylating the Na<sup>+</sup>/H<sup>+</sup> exchanger. The results demonstrate that MAPK-dependent pathways including p90<sup>rsk</sup> and ERK1/2 and are important in regulating the Na<sup>+</sup>/H<sup>+</sup> exchanger and show their dramatic increase in activity toward the Na<sup>+</sup>/H<sup>+</sup> exchanger during ischemia and reperfusion of the myocardium. They also show that ischemia followed by reperfusion have important inhibitory effects on Na<sup>+</sup>/H<sup>+</sup> exchanger activity.**

The Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1 (NHE1)<sup>1</sup> is an integral membrane protein that exchanges one intracellular proton for one extracellular sodium ion in response to intracellular acidification, thereby regulating internal pH (pH<sub>i</sub>) in most mammalian cells (1, 2). Of the six known isoforms of the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE1–NHE6), NHE1 is the major isoform present in the myocardium (3). Ionic homeostasis is vital for normal myocardial function and the regulation of exchanger activity in

the heart by hormones and growth factors via protein kinase-mediated signal transduction, plays an important role in maintenance of this homeostasis (3, 4). The NHE1 protein is ~815 amino acids in length and is thought to consist of a membrane domain with 12 integral membrane segments and a long intracellular carboxyl terminus of 315 amino acids. It is within this intracellular carboxyl terminus that regulation of exchanger activity is postulated to occur by phosphorylation on distal serine/threonine residues (1, 2). Recently, serine 703 has been identified as one important amino acid which is phosphorylated by the protein kinase p90<sup>rsk</sup> (5, 6). However, earlier work has demonstrated growth factor induced phosphorylation of several different peptides of the carboxyl-terminal region of the Na<sup>+</sup>/H<sup>+</sup> exchanger (7, 8). We have also shown that MAPK (ERKs) are involved in hormonal regulation of activity of the exchanger in skeletal muscle (9) and more recently we have shown that both MAPK (ERKs) and p90<sup>rsk</sup> are involved in regulation of exchanger activity in the healthy rat myocardium (10). Another protein kinase, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaM kinase II) has been shown to phosphorylate the COOH-terminal domain *in vitro* (11), but it is not known if CaM kinase II directly regulates the exchanger activity in intact cells.

It is well known that the activity of the Na<sup>+</sup>/H<sup>+</sup> exchanger during ischemia and reperfusion produces numerous secondary effects that lead to the exacerbation of tissue injury. The Na<sup>+</sup>/H<sup>+</sup> exchanger removes protons either during ischemia or during reperfusion which causes excess intracellular Na<sup>+</sup>. This results in either inhibition of the Na<sup>+</sup>/Ca<sup>2+</sup> exchangers ability to extrude Ca<sup>2+</sup>, or reversal in activity of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger and accumulation of Ca<sup>2+</sup>. The increased intracellular Ca<sup>2+</sup> results in a variety of detrimental effects to the heart. It has been shown that inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger activity, via the use of potent amiloride analogs as well as more recently developed NHE1-specific inhibitors, can prove beneficial to recovery from ischemia and reperfusion events (4, 12, 13). Various lines of evidence suggest that the Na<sup>+</sup>/H<sup>+</sup> exchanger may be in an activated state during ischemia and/or reperfusion of the myocardium. α<sub>1</sub>-Adrenergic receptor stimulation regulates the antiporter in the myocardium causing both an alkalization of steady state intracellular pH (pH<sub>i</sub>) and an enhanced rate of Na<sup>+</sup>/H<sup>+</sup> exchanger mediated recovery from an acid load (14–16). α<sub>1</sub>-Adrenergic stimulation is known to exacerbate reperfusion induced arrhythmias and increased Na<sup>+</sup>/H<sup>+</sup> exchanger activity may play an important role in this phenomenon (17). In addition, the 21-amino acid vasoactive peptide endothelin (ET-1) has also been shown to stimulate Na<sup>+</sup>/H<sup>+</sup> exchange in cardiac myocytes (18, 19). Under some circumstances endothelin may also aggravate ischemic reperfusion injury, possibly through Na<sup>+</sup>/H<sup>+</sup> exchanger activation (20).

The protein kinase pathways that could be involved in regulation of the antiporter during ischemia and ischemia reper-

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<sup>1</sup> The abbreviations used are: NHE1, Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1; CaM kinase II, calmodulin dependent protein kinase II; ERK1/2, extracellular signal-regulated kinase 1 and 2; JNK1/2, c-Jun NH<sub>2</sub>-terminal kinase 1 and 2; GST, glutathione S-transferase; MAPK, mitogen activated protein kinase; MEK1/2, MAPK kinase 1 and 2; pH<sub>i</sub>, intracellular pH; IGKA, in-gel kinase assay; KRH, Krebs-Ringer-HEPES; PAGE, polyacrylamide gel electrophoresis.

fusion are not known. It is known that multiple subfamilies of the mitogen-activated protein kinase (MAPK) superfamily such as extracellular signal-regulated kinases (ERKs), p38<sup>MAPK</sup>, and c-Jun NH<sub>2</sub>-terminal kinases (JNKs) are activated in ischemic-reperfused hearts *in vivo* (21, 22). In contrast, some studies only implicate p38 and JNK (stress kinases) activation and not ERKs during ischemia, ischemia reperfusion, oxidative damage, and other stresses to the heart (23–26). However, it has been shown that not only can JNKs and p38 be activated during ischemia reperfusion, but MAPK (ERKs) can also be activated (27). Recently it has been shown that cardiac myocytes exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) show increased Na<sup>+</sup>/H<sup>+</sup> exchanger activity via activation of MAPK-dependent pathways (ERK1/2) (28). It is clearly possible that these signaling pathways may play one or more roles in regulation of the exchanger under ischemia and reperfusion conditions especially since MAPK-dependent pathways have been shown to be important in regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger in the myocardium (10).

To date, no studies have shown definitively which kinases from the ischemic and reperfused heart phosphorylate the distal amino acids in the carboxyl terminus of the Na<sup>+</sup>/H<sup>+</sup> exchanger. The purpose of our study was to examine regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger in the myocardium during ischemia and reperfusion. We examined the ability of multiple protein kinases from ischemic and ischemia-reperfused myocardium to phosphorylate the cytoplasmic domain of the Na<sup>+</sup>/H<sup>+</sup> exchanger. This present study provides the first evidence of protein kinase-mediated regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger in response to ischemia and ischemia reperfusion. The results also suggest that regulation of the myocardial Na<sup>+</sup>/H<sup>+</sup> exchanger may be important in mitigating the damaging effects that ischemia and reperfusion have on the myocardium.

#### EXPERIMENTAL PROCEDURES

**Materials**—PD98059, a MEK1 inhibitor, and SB202190, a p38 inhibitor, were from Calbiochem-Novabiochem Corp. (La Jolla, CA). Plasmid pGEX-3X, glutathione-Sepharose 4B affinity column, and protein A-Sepharose CL-4B beads were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Protein G-PLUS agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK-1/2 (rabbit polyclonal), anti-phospho-ERK (mouse monoclonal), anti-RSK1 (goat polyclonal), anti-p38 (rabbit polyclonal), anti-CaM kinase II (goat polyclonal), anti-JNK2 (rabbit polyclonal), and anti-MEK1 (rabbit polyclonal) were from Santa Cruz Biotechnology. Anti-RSK (p90<sup>RSK</sup>) (mouse monoclonal) was from Transduction Laboratories (Lexington, KY). MF-20 (mouse monoclonal) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). [<sup>32</sup>P]ATP was purchased from Amersham Pharmacia Biotech (Oakville, Ontario, Canada). Collagenase Type 2 was obtained from Worthington Biochemical Corp. (Lakewood, NJ) and BCECF-AM was from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade and were purchased from Fisher Scientific (Ottawa, ON), Sigma, or BDH (Toronto, ON).

**Construction and Purification of Glutathione S-Transferase-Na<sup>+</sup>/H<sup>+</sup> Exchanger Fusion Protein**—The carboxyl-terminal 178 amino acids of the rabbit cardiac Na<sup>+</sup>/H<sup>+</sup> exchanger were expressed as described previously (9) as a fusion protein with GST (PCRA) using the plasmid pGEX-3X. The *Escherichia coli* TOPP 2 strain was induced with 1 mM isopropylthio-β-D-galactoside. GST-Na<sup>+</sup>/H<sup>+</sup> exchanger fusion protein was purified via glutathione-Sepharose 4B affinity chromatography as described earlier (9, 10).

**Animals and Ischemic Reperfusion of Hearts**—Adult Harlan Sprague-Dawley rats were purchased from either Charles River Canada (St. Constant, PQ) or Harlan Sprague-Dawley (Indianapolis, IN) and maintained in the Health Sciences Animal Care facility of the University of Western Ontario in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, ON). Rats were killed by decapitation and the hearts were immediately removed and perfused by way of the aorta at 37 °C with oxygenated Krebs-Henseleit buffer consisting of 120 mM sodium chloride, 4.63 mM potassium chloride, 1.17 mM potassium phosphate monobasic, 1.25 mM calcium chloride, 1.2 mM magnesium chloride, 20.0 mM sodium bicarbonate, and 8.0 mM glucose,

pH 7.4, at a flow rate of 10 ml/min (29). All hearts were equilibrated for 30 min, after which ischemia was induced for 30 min by completely stopping the flow with or without subsequent reperfusion for 30 min. Control hearts were perfused normally for 60 min. In another set of experiments, hearts were allowed to equilibrate for 10 min. This was followed by 30 min of buffer perfusion with or without 40 μM PD98059 (a MAPK kinase (MEK1) inhibitor), then 30 min of no flow (ischemia), followed by 15 min of reperfusion with or without 40 μM PD98059. At the end of the perfusions hearts were “freeze clamped” and stored at minus 80 °C.

**Preparation of Cell Extracts from Control, Ischemic, and Ischemic-reperfused Adult Rat Myocardium**—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 50 mM tetrasodium pyrophosphate, 50 mM sodium fluoride, 50 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 10 mM HEPES, pH 7.4, and a mixture of protease inhibitors. The homogenate was centrifuged at 6,000 × g × 60 min at 4 °C. The supernatant was then centrifuged at 10,000 × g × 60 min at 4 °C. Western blot analysis was done using commercially available antibodies against ERK 1/2, phospho-ERK, p38, JNK2, p90<sup>RSK</sup>, MEK1, and MF-20 (cardiac sarcomeric myosin heavy chain). Ischemic-reperfused hearts were also perfused in the presence of the drug PD98059, to partially inhibit the MAPK (ERK1/2) pathway by inhibiting MEK1. Heart cell extracts from these experiments were prepared as above.

**In-gel Kinase Assays**—To identify protein kinases that phosphorylated the Na<sup>+</sup>/H<sup>+</sup> exchanger fusion protein, cell extracts (80 μg of protein) from control, ischemic, and ischemic-reperfused myocardium (with and without 40 μM PD98059) were separated by 10% SDS-PAGE in a gel containing 1 mg/ml substrate. In-gel kinase assays were performed as previously described (10). The gels were dried for autoradiography and visualization of phosphorylation. Equal amounts of protein were 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 MF-20. Protein concentrations were measured using the Bio-Rad DC protein assay. For one series of experiments samples in IGKAs were treated with 5 μM SB202190 during incubation with labeled ATP to inhibit p38 kinase.

**Immunoprecipitation of ERK1/2, p90<sup>RSK</sup>, p38, JNK2 and MEK1 from Control, Ischemic, and Ischemic-reperfused Heart Cell Extracts**—For immunoprecipitations, ERK-1/2, p38, RSK1, JNK2, and MEK1 antibodies were used. Extracts (1 ml) were pretreated by incubating with protein A-Sepharose CL-4B beads (in experiments with ERK-1/2, anti-p38, JNK2, and MEK1 antibodies) or protein G-PLUS agarose beads (in experiments with anti-RSK1) for 30 min. The samples were then 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. The beads were then incubated with heart cell extract for 2 h at 4 °C and washed with extraction buffer. For immunoprecipitation of protein kinases, 30 μl of 200 μg/ml stock anti-ERK-1/2, MEK1, JNK2, or anti-p38 was added to pretreated heart cell extract and this mixture was rotated for 2 h at 4 °C. The antibody-antigen complex was then added to pretreated protein A-Sepharose beads and rotated for 1 h at 4 °C. The beads were next washed extensively in extraction buffer and solubilized in SDS-PAGE gel sample buffer. For the remaining immunoprecipitation, 10 μl of 200 μg/ml stock anti-RSK1 was added to protein G-PLUS agarose beads overnight at 4 °C. The next day the heart cell extract was added to this antibody-bead complex and allowed to bind for 4 h at 4 °C. The beads were then washed extensively in extraction buffer and solubilized in SDS-PAGE gel sample buffer. The immunoprecipitates were all analyzed by Western blotting and by in-gel kinase assay for their ability to phosphorylate the Na<sup>+</sup>/H<sup>+</sup> exchanger fusion protein.

**Primary Cultures of Isolated Neonatal Myocytes**—Primary myocyte cultures were prepared from neonatal Harlan Sprague-Dawley rat heart ventricles as described previously (10). Isolated primary myocytes were plated onto glass coverslips for physiologic studies, or onto Primaria<sup>TM</sup> (Falcon) culture dishes or flasks for collection of cell extracts. Myocytes were maintained for 4–5 days in medium containing Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 10% fetal bovine serum, 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 non-essential amino acids, 10% MEM vitamin, 0.1 mM bromodeoxyuridine, 100 μM L-ascorbic acid, and 30 mM HEPES, pH 7.1. Cells were serum-starved overnight prior to all experiments.



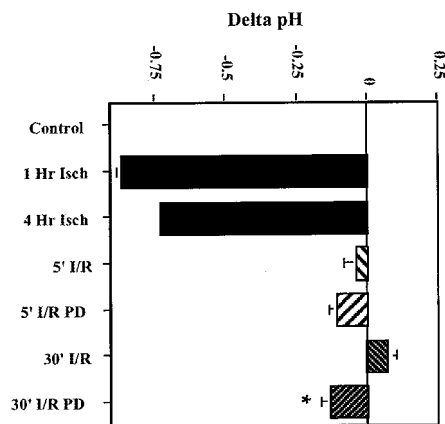
**Ischemia and Ischemia Reperfusion Conditions in Cardiac Myocytes**—Neonatal cardiac myocytes were made ischemic by incubation at pH 6.2 in Krebs-Ringer-HEPES (KRH) buffer containing 130 mM sodium chloride, 2.5 mM potassium chloride, 2.5 mM potassium cyanide (to inhibit oxidative phosphorylation), 1 mM potassium phosphate monobasic, 1.2 mM magnesium sulfate, 2 mM calcium chloride, and 20 mM HEPES for 4 h at 37 °C (modified from Bond *et al.* (30)). This ischemic treatment is referred to as **I1**. In other experiments, 10 mM 2-deoxy-D-glucose was added to this medium to inhibit glycolytic ATP production and result in a more severe ischemia (referred to as **I2**) (30). To simulate reperfusion, the metabolic inhibitor was removed by “washout” with KRH buffer at pH 7.4 (without potassium cyanide) and incubation for 30 min at 37 °C. Control myocytes were incubated with KRH buffer, pH 7.4 (without potassium cyanide), for the same time period as the ischemic myocytes. Following the experiment, 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, 0.1% Triton X-100, 10 mM HEPES, pH 7.4, and a mixture of protease inhibitors (31). The cells were then 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. Protein kinases from the extracts were analyzed by Western blot analysis using antibodies against ERK 1/2, phospho-ERK, and MF-20 (cardiac sarcomeric myosin heavy chain) and for their ability to phosphorylate the Na<sup>+</sup>/H<sup>+</sup> exchanger fusion protein using the in-gel kinase assay described earlier.

**Measurement of Intracellular pH during Ischemia and Ischemia Reperfusion Conditions in Isolated Neonatal Cardiac Myocytes**—Myocytes were grown on glass coverslips and the acetoxymethyl ester of 2'-7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein was used to measure steady-state pH<sub>i</sub> as described previously (9, 32). Briefly, pH<sub>i</sub> was measured using the dual excitation single emission ratio technique using a temperature-controlled Shimadzu RF5000 spectrofluorophotometer. Excitation wavelengths were at 452 and 503 nm with emission at 524 nm. Myocytes were serum-starved before all pH<sub>i</sub> measurements. Cells on coverslips were placed into a holder device and inserted into a 1 × 1-cm fluorescence cuvette at 37 °C and bathed in the ischemia and reperfusion (washout) buffers as stated above (KRH buffer at pH 6.2 or 7.4, respectively). Steady-state pH<sub>i</sub> was measured for 1 min after the specified ischemia or ischemia-reperfusion protocol (see above). In some experiments, the cells were pretreated with the MEK1 inhibitor PD98059 (50 μM) during the last 30 min of ischemia and during the reperfusion period. PD98059 was added during the reperfusion phase and at all subsequent steps. For other experiments, SB202190 (1 μM) was added during the ischemic phase as well as at the reperfusion phase.

Rates of recovery from an acid load in control, ischemic, and ischemia-reperfused neonatal cardiac myocytes (with and without PD98059 or SB202190) were also measured following an acid challenge using the ammonium chloride prepulse method as previously described (9, 32). Cells were treated either with an ischemia induced by using KCN only (**I1**) or with a harsher form of ischemia induced by using KCN and 2-deoxy-D-glucose (**I2**). Briefly, ammonium chloride (20 mM) was added for 4 min followed by a pH recovery that was obtained by transferring the cells to buffer containing 130 mM sodium chloride after a brief exposure to a Na<sup>+</sup>-free buffer. For measuring initial rate of recovery from an acid load we examined the initial changes in pH<sub>i</sub> during the first 10–20 s after sodium chloride addition.

We calculated proton flux for all experiments involving rate of recovery from an acid load. Buffering capacity (B, millimole/liter/pH unit) was estimated as the amount of acid loaded divided by the observed change of cell pH<sub>i</sub> produced by this load. Buffering capacity was determined at various pH<sub>i</sub> by varying amounts of ammonium chloride. Proton flux ( $J_{H^+}$ ) produced due to the Na<sup>+</sup>/H<sup>+</sup> exchanger after acid loading was calculated from the buffer capacity at mid point pH × ΔpH/Δt time essentially as described earlier (33).

**Data Analysis**—Autoradiographs were quantified using a model BAS1000 PhosphorImager (Fuji Photo Film Co., Ltd) to examine radioactivity incorporated into protein (in-gel kinase assay). Analysis of these results was by Mann-Whitney *U* test and/or Student's unpaired *t* test on direct numerical values obtained from the image analysis. Quantification and analysis of results of pH<sub>i</sub> measurements was done using a Mann-Whitney *U* test. Values presented are mean ± S.E. with at least four and up to eight experiments being done in every case; *p* values < 0.05 were considered statistically significant.

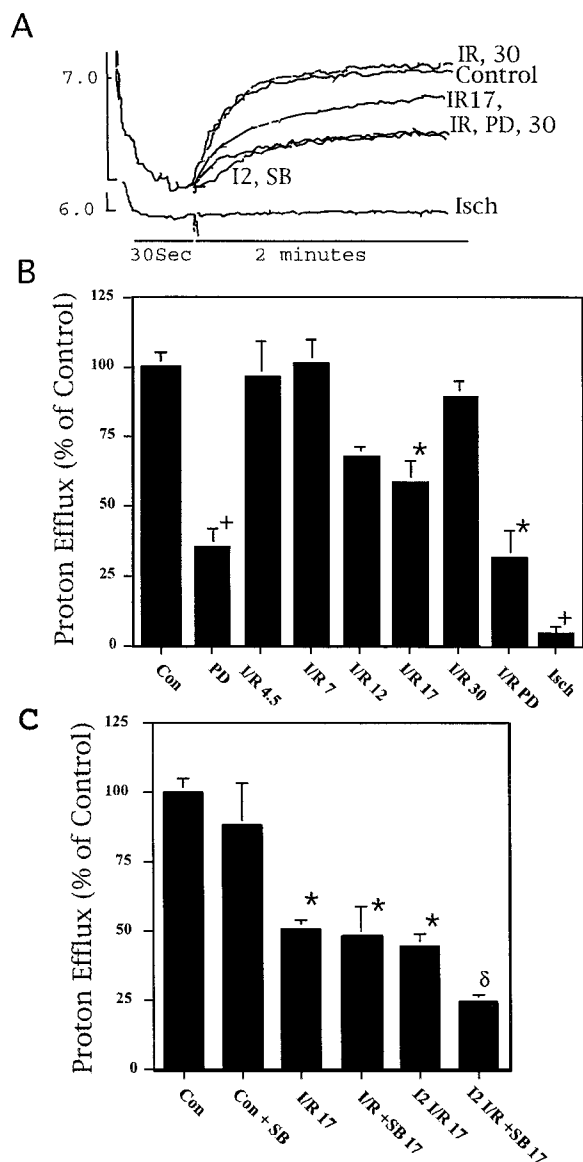


**FIG. 1. Effect of ischemia and ischemia reperfusion conditions on steady-state pH<sub>i</sub> of isolated neonatal ventricular myocytes.** Myocytes were grown on coverslips and serum-starved overnight prior to stimulation in the presence or absence of 50 μM PD98059. Ischemia (**I1**) and reperfusion were for the indicated times. Results are mean ± S.E. of at least four experiments. Where error bars are absent they were too small to be displayed. Asterisk (\*) indicates that the value in the presence of PD98059 is significantly different from the untreated value at *p* < 0.05.

## RESULTS

**A MAPK (ERK)-dependent Pathway Facilitates Steady-state pH<sub>i</sub> Regulation by the Na<sup>+</sup>/H<sup>+</sup> Exchanger in Response to Ischemia Reperfusion**—To observe the regulation of the endogenous Na<sup>+</sup>/H<sup>+</sup> exchanger, the effect of ischemia and ischemia reperfusion on steady-state pH<sub>i</sub> of neonatal cardiac myocytes was examined. Isolated neonatal cardiac myocytes were grown on coverslips and serum-starved overnight prior to stimulation. We utilized methods that “chemically” induce ischemia and ischemia-reperfusion events similar to that observed in perfused hearts. This “chemical” ischemia acts by inhibition of oxidative phosphorylation (**I1**), or by inhibiting both oxidative phosphorylation and glycolysis (**I2**) (30). Fig. 1 summarizes the effects of ischemia (**I1**) and ischemia reperfusion on steady-state pH<sub>i</sub> in the presence or absence of the MEK1 inhibitor 50 μM PD98059. Ischemia reduced steady-state pH<sub>i</sub> dramatically while 5 and 30 min of reperfusion brought about a complete recovery of normal pH<sub>i</sub> (to ~7.2). Treatment of the myocytes with 50 μM PD98059 significantly reduced the ability of the cells to return pH<sub>i</sub> to the values observed without PD98059. This indicated that a MAPK (ERK) pathway is partially responsible for facilitating Na<sup>+</sup>/H<sup>+</sup> exchanger activity during ischemia reperfusion in neonatal ventricular myocytes. PD98059 alone had no effect on resting pH<sub>i</sub> of isolated myocytes that had not undergone ischemia or reperfusion (not shown).

**A MAPK (ERK)-dependent Pathway Facilitates the Rate of Recovery from Acid Load Induced by Ischemia Reperfusion**—To observe changes in proton efflux during ischemia and ischemia reperfusion, an ammonium chloride prepulse was used to induce acute acidosis in control myocytes and in various groups of myocytes treated with ischemia (**I1** or **I2**) as described for Fig. 1. Three groups were examined. Control cells were treated with KRH buffer for 30 min to 4 h and then treated with ammonium chloride. This was followed by a brief period in Na<sup>+</sup>-free KRH buffer and then recovery in KRH buffer with normal Na<sup>+</sup>. These cells recovered rapidly from the acid load (Fig. 2A). A second group of cells (Isch) was given the same kind of treatment except that at all stages the KRH buffer was replaced either with KCN containing buffer (**I1**) or buffer used to induce chemical ischemia. These cells retained a much more acidic pH<sub>i</sub> at all stages and did not recover from an acid load (Fig. 2, A and B). The third group of cells (I/R) was subject to 3–4 h of



**FIG. 2. Recovery of  $pH_i$  from acute acid load by isolated neonatal ventricular myocytes after ischemia and ischemia reperfusion in the presence or absence of 50  $\mu M$  PD98059 or 1  $\mu M$  SB202190.** Ischemia was induced by the presence of KCN (2.5 mM) or KCN plus 2-deoxy-D-glucose (10 mM) where indicated (**I2**). Ammonium chloride prepulse was used to induce acute acidosis. Control cells were treated 30 min to 4 h with KRH, followed by ammonium chloride prepulse, and a brief period in  $Na^+$ -free KRH buffer and recovery in KRH buffer with  $Na^+$ . *I/R* (ischemia-reperfusion) cells were treated 3–4 h with chemical ischemia buffer (as described under “Experimental Procedures”) followed by various incubations in normal KRH buffer (with or without PD98059 (A and B) or SB202190 (A and C)). Ammonium chloride prepulse and recovery measurement followed as with controls. The inhibitors PD98059 and SB202190 were present in all solutions used for  $pH_i$  recovery measurements. A, illustrates representative tracings of  $pH_i$  recovery of myocytes. B and C, bar graphs summarize the results of initial rate of  $pH_i$  recovery from an acute acid load. Results are mean  $\pm$  S.E. of at least four experiments. Plus and asterisk (+, \*) indicate significant difference from control values at  $p < 0.01$  and  $p < 0.05$ , respectively.  $\delta$  indicates significant difference from no SB202190 treatment at  $p < 0.01$ .

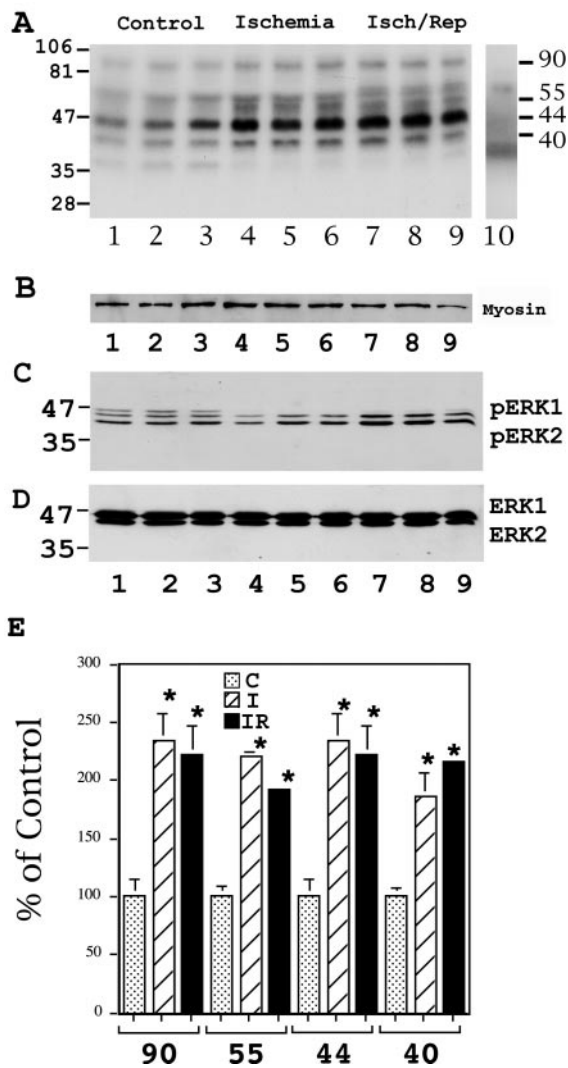
chemical ischemia (either **I1** or **I2**), followed by varying reperfusion periods (4.5–30 min) in normal KRH buffer (with or without PD98059 (**I1**) or SB202190 (**I1** and **I2**)). They were then subjected to ammonium chloride-induced acid load and recovery in normal KRH buffer (with or without PD98059 or SB202190). Cells treated with PD98059 recovered much more slowly from the acid load in comparison to untreated cells and

reached a lower steady state  $pH_i$  (Fig. 2, A and B). There was no difference in the rate of recovery from an acid load when comparing PD98059-treated control cells and PD98059-treated cells subjected to ischemia and reperfusion (Fig. 2B). Ischemia (**I1**) followed by reperfusion resulted in a large (up to 50%) reproducible, time-dependent decrease in the ability of cells to recover from an acid load. At both 12 and 17 min of reperfusion there was a decrease in the ability of cells to recover from acid load (Fig. 2, A–C). This effect largely disappeared after 30 min of reperfusion.

The effect of SB202190 on rate of recovery from an acute acid load is shown in Fig. 2C. Control cells treated with SB202190 showed only a slight, variable reduction in the rate of recovery from acid load. Isolated myocytes were treated with ischemia (**I1**) followed by reperfusion for 17 min. SB202190 had no effect on the ability of these **I1**-treated cells to recover from an acid load. When isolated myocytes were treated with the harsher form of ischemia (**I2**) followed by reperfusion for 17 min, SB202190 slightly (but significantly) reduced the ability of these cells to recover from an acid load. SB202190 had no effect on the steady state resting  $pH_i$  of isolated myocytes (not shown).

*Protein Kinases Phosphorylating the  $Na^+/H^+$  Exchanger Are Activated by Ischemia and Reperfusion in Isolated Neonatal Ventricular Myocytes*—We then examined the effect of ischemia and ischemia reperfusion on phosphorylation of the  $Na^+/H^+$  exchanger COOH terminus in isolated, neonatal cardiac myocytes. Cells were maintained either under control conditions (Krebs-Ringer-HEPES (KRH) buffer, pH 7.4, Fig. 3, lanes 1–3), subjected to ischemia (**I1**) for 4 h (KRH buffer + 2.5 mM KCN, pH 6.2, lanes 4–6) or subjected to ischemia followed by reperfusion or washout for 30 min with the control KRH buffer (lanes 7–9). Samples of myocyte extracts were used in an in-gel kinase assay with the  $Na^+/H^+$  exchanger as a substrate as described earlier (10). Four major kinases of approximate molecular masses 90, 55, 44, and 40 kDa were activated during both ischemia and ischemia reperfusion of neonatal myocytes. In-gel kinase analysis with only GST as a substrate did not give any of these major bands and usually gave only weak activity at sizes of  $\sim 70$  and 37 kDa. Based on the size of the protein kinases phosphorylating the  $Na^+/H^+$  exchanger on our previous study (10) and on Western blot analysis of the same samples (Fig. 3, C and D), we tentatively identified ERK1/2 (44 and 42-kDa) and  $p90^{rsk}$  (90-kDa) as the 44- and 90-kDa protein kinases that were present in the cell extracts. The two remaining kinases of molecular masses 40 and 55 kDa remained to be identified. Increasing amounts of phosphorylation of the  $Na^+/H^+$  exchanger was observed by all four of the major protein kinases activated by ischemia and ischemia reperfusion (Fig. 3, A and E). Western blot analysis with antibodies to MF-20 (Fig. 3B) and ERK1/2 (Fig. 3D) confirmed that equal amounts of protein were present in control versus treated myocytes. Immunoblotting with anti-phospho ERK1/2 (Fig. 3C, lanes 7–9) show increased activation of both these kinases during ischemia followed by reperfusion. Qualitatively similar results were obtained with extracts of cells from myocytes treated with the more severe ischemia (**I2**).

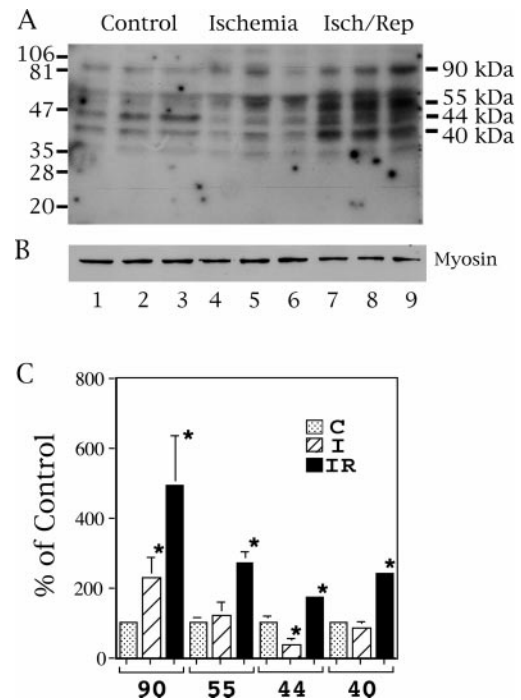
In another set of experiments (Fig. 4) we used a similar approach to examine the effects of more severe ischemia (**I2**) and reperfusion on phosphorylation of the  $Na^+/H^+$  exchanger COOH terminus in isolated, neonatal cardiac myocytes. Using a similar protocol we found the same proteins phosphorylated in control isolated myocytes (Fig. 4, lanes 1–3). Ischemia and *I/R* resulted in large increases in the level of phosphorylation of the 90-kDa band. There was no increase in the level of the 55-, 44-, or 40-kDa band in ischemia alone, however, for all these



**FIG. 3.  $\text{Na}^+/\text{H}^+$  exchanger-directed protein kinase activity of cell extracts from ischemic-reperfused isolated neonatal ventricular myocytes.** Cells were maintained either under control conditions (KRH buffer pH 7.4, lanes 1–3), subjected to ischemia (I) for 4 h in KRH buffer + 2.5 mM KCN, pH 6.2 (lanes 4–6), or subjected to ischemia followed by reperfusion or washout for 30 min with the control KRH buffer (lanes 7–9). Myocyte extracts were used for: A, in-gel kinase assay as described under “Experimental Procedures.” Lane 10 is a myocyte extract run in a gel using GST alone as a substrate (1 mg/ml). B–D, Western blots of simultaneously run samples (1–9) that were used in A. The Western blots were probed with antibodies to MF-20, phospho-ERK1/2, and ERK1/2 for B–D, respectively. E, bar graph summarizing results in A. Asterisk (\*) indicates significant difference from control values at  $p < 0.05$ .

kinases, the activity was significantly increased by reperfusion following ischemia. The 90-kDa kinase was most strongly activated by the severe ischemia and reperfusion treatment. The level of phosphorylation by the 44-kDa kinase was decreased from the control levels by ischemia alone.

**Ischemia and Ischemia Reperfusion of the Intact Rat Myocardium Activate Multiple Protein Kinases That Phosphorylate the Carboxyl-terminal of the  $\text{Na}^+/\text{H}^+$  Exchanger**—In-gel kinase assay and Western analysis were used to identify potential protein kinases that phosphorylated the carboxyl-terminal of the  $\text{Na}^+/\text{H}^+$  exchanger during ischemia and ischemia reperfusion of the intact adult rat myocardium. Samples of heart cell extracts (Fig. 5A, controls, lanes 1–3; ischemic, lanes 4–6; ischemia-reperfused, lanes 7–9) were used in in-gel kinase assays as described under “Experimental Procedures.” Four major kinases of approximate molecular masses 90, 55, 44, and 40

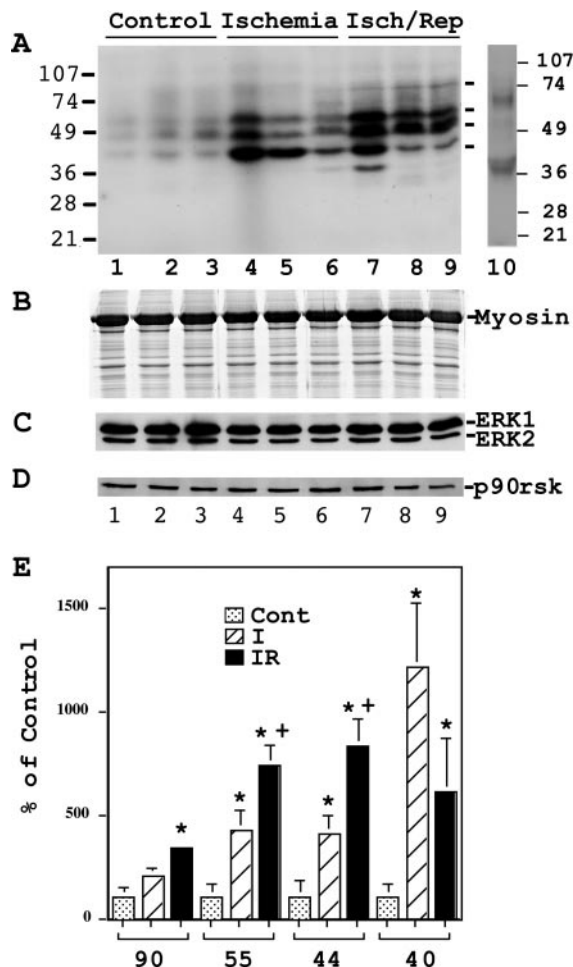


**FIG. 4.  $\text{Na}^+/\text{H}^+$  exchanger-directed protein kinase activity of cell extracts from ischemic-reperfused isolated neonatal ventricular myocytes.** Cells were maintained either under control conditions (KRH buffer pH 7.4, lanes 1–3), subjected to severe ischemia (I) for 4 h in KRH buffer + 2.5 mM KCN, pH 6.2, and 10 mM 2-deoxyglucose (lanes 4–6), or subjected to ischemia followed by reperfusion or washout for 30 min with the control KRH buffer (lanes 7–9). Myocyte extracts were prepared as described in the legend to Fig. 3. Panel B is a Western blot of a simultaneously run sample, probed with an antibody to MF-20. C, bar graph summarizing results in A. Asterisk (\*) indicates significant difference from control values at  $p < 0.05$ .

kDa were able to significantly phosphorylate the carboxyl-terminal region of the  $\text{Na}^+/\text{H}^+$  exchanger. Control experiments with GST alone (not shown) as a substrate did not show phosphorylation of the kinases of approximate molecular masses 90, 55, 44, and 40 kDa. The four kinases were all activated during both ischemia and ischemia reperfusion of the rat myocardium in comparison to control hearts. For the 90- and 55-kDa protein kinases the phosphorylation was increased by reperfusion of the hearts in comparison to the effect of ischemia alone. With the 40- and 90-kDa protein kinases reperfusion caused further activation of the kinases (Fig. 5E).

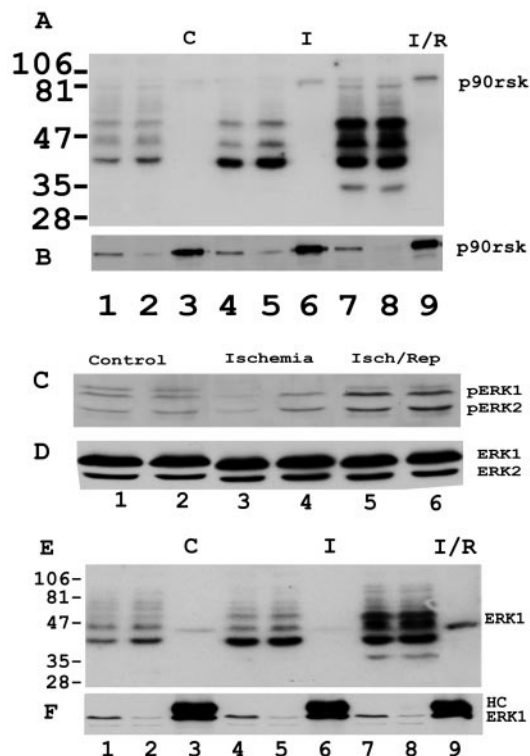
**Immunoprecipitated ERK1/2 and  $p90^{\text{rsk}}$  Phosphorylate the  $\text{Na}^+/\text{H}^+$  Exchanger during Ischemia and Ischemia Reperfusion of the Rat Myocardium**—To more conclusively identify ERK1/2 and  $p90^{\text{rsk}}$  (RSK1) as activated protein kinases that phosphorylate the carboxyl-terminal of the  $\text{Na}^+/\text{H}^+$  exchanger, these kinases were immunoprecipitated with antibodies specific to ERK1/2 and  $p90^{\text{rsk}}$  (RSK1). Specific kinases were immunoprecipitated from cell extracts of control (Fig. 6, lanes 1–3), ischemia (lanes 4–6), and ischemic-reperfused (lanes 7–9) hearts and the immunoprecipitates were used in an in-gel kinase analysis (Fig. 6, A and E). The first two lanes for each group are before and after immunoprecipitation while the third lane is the immunoprecipitate. Western blots of the samples confirmed the presence of equal amounts of immunoprecipitated protein for each of the different groups and confirmed the identity of the immunoprecipitates (Fig. 6, B, D, and F). Fig. 6A shows that during ischemia and ischemic reperfusion,  $p90^{\text{rsk}}$  was activated and phosphorylated the  $\text{Na}^+/\text{H}^+$  exchanger more than controls.  $p90^{\text{rsk}}$  was highly activated during ischemia reperfusion in comparison to either control hearts or hearts treated with ischemia alone. A similar result occurred with





**FIG. 5. Characterization of  $\text{Na}^+/\text{H}^+$  exchanger-directed protein kinase activity of cell extracts from isolated perfused rat hearts.** Heart extracts were prepared from control isolated perfused hearts (lanes 1–3), from hearts subjected to 30 min ischemia (lanes 4–6), and from hearts subjected to 30 min ischemia followed by 30 min reperfusion (lanes 7–9). **A**, autoradiogram showing in-gel kinase assay using  $\text{Na}^+/\text{H}^+$  exchanger-GST as substrate (1 mg/ml, lanes 1–9) and an ischemia-reperfused sample using GST protein as substrate (1 mg/ml, lane 10). Right of lane 9 indicates the 90-, 55-, 44-, and 40-kDa protein kinases. **B**, Coomassie Blue-stained SDS-PAGE gel of identical samples (lanes 1–9) noting the position of cardiac sarcomeric myosin heavy chain protein. **C**, Western blot analysis of the same samples using anti-ERK1 (rabbit polyclonal, 1:4000) antibody to detect ERK1/2 (44 and 42 kDa) in cell extracts. **D**, Western blot using anti-RSK (p90<sup>rsk</sup>) (mouse monoclonal, 1:500) to detect the presence of this kinase in the heart cell extracts. **E**, bar graph summarizing the effects of ischemia and ischemia reperfusion on exchanger-directed protein kinase activity of 4 protein kinases (90, 55, 44, and 40 kDa) present in heart cell extracts. Results are mean  $\pm$  S.E. of three experiments. Asterisk (\*) indicates significant difference from control value at  $p < 0.05$ . Plus (+) indicates significant difference from ischemic value at  $p < 0.05$ .

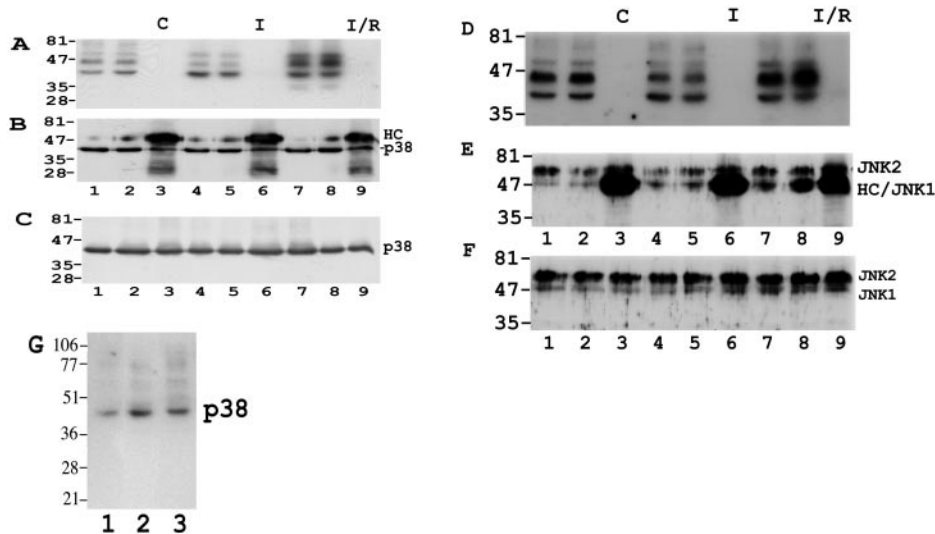
immunoprecipitated ERK1 (Fig. 6, *E* and *F*). ERK1 was successfully immunoprecipitated from control, ischemic, and ischemia-reperfused tissue (lanes 3, 6, and 9, respectively). Western analysis with anti-ERK1 antibody confirmed the identity of the immunoprecipitates (Fig. 6*F*). The in-gel kinase assay showed that ERK1 was highly activated during ischemia reperfusion and phosphorylated the  $\text{Na}^+/\text{H}^+$  exchanger fusion protein more than both control and ERK1 immunoprecipitates from hearts treated with ischemia. Western blot analysis with anti-phospho-ERK1 antibody confirmed that ERK1 was highly activated by ischemia followed by reperfusion (Fig. 6*C*). ERK2 was also weakly activated during ischemia reperfusion and phosphorylated PCRA, but to a much lesser degree (data not shown). It appears that both ERK1 and -2 are phosphorylated



**FIG. 6. In-gel kinase assays demonstrating activated p90<sup>rsk</sup> (RSK1) and ERK1-mediated phosphorylation of the  $\text{Na}^+/\text{H}^+$  exchanger.** Cell extracts were prepared from the various isolated perfused rat hearts as described under "Experimental Procedures." p90<sup>rsk</sup> (RSK1) (**A**) and ERK1 (**E**) antibodies were used to immunoprecipitate these protein kinases. Samples of immunoprecipitates and cell extracts before and after immunoprecipitation were then used for in-gel kinase assays with  $\text{Na}^+/\text{H}^+$  exchanger protein as substrate. Lanes 1–3 are from control (C) hearts: lane 1, cell extracts before; and lane 2, after immunoprecipitation; lane 3 is the immunoprecipitate. Lanes 4–6 are ischemic (I) samples and lanes 7–9 are ischemic-reperfused (I/R) samples run on the gel using the same loading pattern. **B** and **F** are Western blots of simultaneously run samples that were used in **A** and **E**, respectively. They were probed with antibodies to p90<sup>rsk</sup> (RSK1) and ERK1, respectively. **C** and **D** are Western blots performed with antibodies to the phosphorylated form of ERK1 (mouse monoclonal, 1:1000) and ERK1, respectively (lanes 1 and 2 are control cell extracts; 3 and 4, ischemic cell extracts; and 5 and 6 ischemic-reperfused cell extracts). HC indicates the position of the heavy chain of IgG used for immunoprecipitation and is evident when probing the immunoprecipitate with secondary antibody.

during ischemia followed by reperfusion. However, immunoprecipitation of ERK2 with anti-ERK2 antibodies suggested that ERK2 does not appear to phosphorylate the  $\text{Na}^+/\text{H}^+$  exchanger as strongly as ERK1 (data not shown).

**Activity of p38 and JNK2 in IGKAs Directed Toward the  $\text{Na}^+/\text{H}^+$  Exchanger**—To identify p38 (38-kDa) and/or JNK2 (54-kDa) (stress kinases) as activated protein kinases that phosphorylate the  $\text{Na}^+/\text{H}^+$  exchanger, these kinases were immunoprecipitated with specific antibodies. (Antibody to JNK2 recognizes both JNK1/2 in Western analysis, but only immunoprecipitates JNK 2.) p38 (Fig. 7, *A* and *B*) and JNK2 (Fig. 7, *D* and *E*) were immunoprecipitated from cell extracts from control (Fig. 7, lanes 1–3), ischemic (lanes 4–6), and ischemia-reperfused (lanes 7–9) hearts. The immunoprecipitates were used in an in-gel kinase analysis (p38, Fig. 7*A*; JNK, Fig. 7*D*). Western blot analysis of the samples confirmed the presence of immunoprecipitated protein for each type of cell extract (Fig. 7, *B* and *E*). Fig. 7, *C* and *F*, also confirm the presence of p38 and JNK1/2 in control (lanes 1–3), ischemia (lanes 4–6), and ischemic reperfusion (lanes 7–9) hearts. Fig. 7*A* shows that there was no phosphorylation of the  $\text{Na}^+/\text{H}^+$  exchanger by immunopre-



**FIG. 7. In-gel kinase assays demonstrating lack of ability of MAPK family members, p38 (38-kDa) and JNK2 (54-kDa) (stress kinases), to phosphorylate the  $\text{Na}^+/\text{H}^+$  exchanger.** Cell extracts were prepared from the various isolated perfused rat hearts as described for Fig. 1. p38 (A) and JNK2 (D) antibodies were used to immunoprecipitate these protein kinases. Immunoprecipitates were used in in-gel kinase assays with  $\text{Na}^+/\text{H}^+$  exchanger protein as substrate (lanes 1–3 are control (C) cell extract; before immunoprecipitation (lane 1), after immunoprecipitation (lane 2), and the immunoprecipitate is in lane 3). Lanes 4–6 are ischemic (I) samples and lanes 7–9 are ischemic-reperfused (I/R) samples using the same loading pattern as the controls. B and E are Western blots of simultaneously run samples that were used in A and D. They were probed with antibodies to p38 (rabbit polyclonal, 1:1000) and JNK2 (rabbit polyclonal, 1:1000), respectively. C and F are Western blots with antibodies to p38 and JNK2, respectively. They demonstrate the presence of these protein kinases in the three types of treated hearts (lanes 1–3 are control heart extracts; lanes 4–6, ischemic heart extracts; and lanes 7–9, ischemic-reperfused heart extracts). HC indicates the position of the heavy chain of IgG used for immunoprecipitation. G, in-gel kinase assay showing p38 protein kinase from control (lane 1), ischemic (lane 2), and ischemic-reperfused (lane 3) heart extracts phosphorylating its ATF-2 substrate.

precipitated p38 from heart extracts. Similarly, Fig. 7D shows no phosphorylation of the  $\text{Na}^+/\text{H}^+$  exchanger by immunoprecipitated JNK2. The results demonstrated that neither our immunoprecipitate of p38 nor that of JNK2, phosphorylate the carboxyl-terminal of the  $\text{Na}^+/\text{H}^+$  exchanger. Fig. 7G confirms that p38 from heart extracts phosphorylates the substrate ATF-2 in an in-gel kinase assay (Fig. 7G). Despite repeated attempts at improvement, the immunoprecipitation of p38 from the isolated myocytes was relatively inefficient, while that from other cells was greatly improved and phosphorylated the  $\text{Na}^+/\text{H}^+$  exchanger very readily (not shown). The reason for this difference is not clear at this time but could be due to differences in isoforms of the p38 kinase that occur in various tissues (34). Our results suggest that either p38 and JNK2 from the myocardium do not phosphorylate the  $\text{Na}^+/\text{H}^+$  exchanger or that their phosphorylation was below the level of detection of this assay.

In another experiment, we examined the effect of SB202190 on the protein kinase activity of heart extracts in an in-gel kinase assay. Prior to incubation with ATP, the gel containing the heart extract was incubated with  $10 \mu\text{M}$  SB202190. There was no reduction of any specific molecular weight protein kinase activity directed toward the  $\text{Na}^+/\text{H}^+$  exchanger, despite a general overall reduction in all protein kinases activity (not shown).

**Inhibition of MEK1 by PD98059 Reduces  $\text{Na}^+/\text{H}^+$  Exchanger Kinase Activation during Ischemia Reperfusion of the Myocardium**—In-gel kinase assay and Western analysis were used to identify differences in protein kinase activation during ischemia reperfusion in the absence (Fig. 8, A and B, lanes 1–3) or presence (lanes 4–6) of  $40 \mu\text{M}$  PD98059, a MEK1 inhibitor. The use of PD98059 reduced the phosphorylation activity of the 90-kDa kinases toward the  $\text{Na}^+/\text{H}^+$  exchanger (Fig. 8, A and C). The use of antibodies to the phosphorylated form of ERK1/2 (Fig. 8B) confirmed that there was a large reduction in activation of ERK1/2 by treatment with PD98059. There was no reduction in the level of phosphorylation by the 40-kDa kinase

and surprisingly, there was also no significant reduction in the amount of phosphorylation by the 44-kDa kinase.

In another series of experiments we postulated that some of the kinase activity we observed might be due to MEK1 (43-kDa) and/or MEK2 (56-kDa). However, in a series of experiments we immunoprecipitated MEK1/2 and examined their ability to phosphorylate the antiporter in in-gel kinase assays. The results showed that phosphorylation by the 55- and 40-kDa protein kinases were not due to MEK1/2 (not shown). A similar experiment with anti-CaM kinase II antibody also showed that the 55-kDa protein kinase was not CaM kinase II (not shown).

## DISCUSSION

It is well established that the activity of the  $\text{Na}^+/\text{H}^+$  exchanger exacerbates the tissue injury that occurs during ischemia and reperfusion of the myocardium (4, 12). However, regulation of the activity of the  $\text{Na}^+/\text{H}^+$  exchanger during this time period has not been studied. To investigate this phenomenon further, we examined protein kinase-mediated regulation of the  $\text{Na}^+/\text{H}^+$  exchanger during ischemia and reperfusion of the myocardium. We initially demonstrate that ERK-dependent pathways are involved in activity of the  $\text{Na}^+/\text{H}^+$  exchanger when recovering from ischemia and reperfusion. When isolated myocytes were exposed to ischemia followed by reperfusion, PD98059 inhibited their ability to return to resting pH. PD98059 also inhibited the ability to recover from an acute acid load, although there was no difference between control hearts treated with PD98059 and hearts that had undergone ischemia and reperfusion. These results suggest that ERK-dependent pathways are important in maintenance of resting pH in the ischemic myocardium and that they are normally important in the ability of the  $\text{Na}^+/\text{H}^+$  exchanger to recover from an acute acid load. Because of the time required for inducing acute acid loading, it was not possible to measure the effects of reperfusion on  $\text{Na}^+/\text{H}^+$  exchanger very early after ischemia. Therefore it was not possible to examine if very early reperfusion elevates

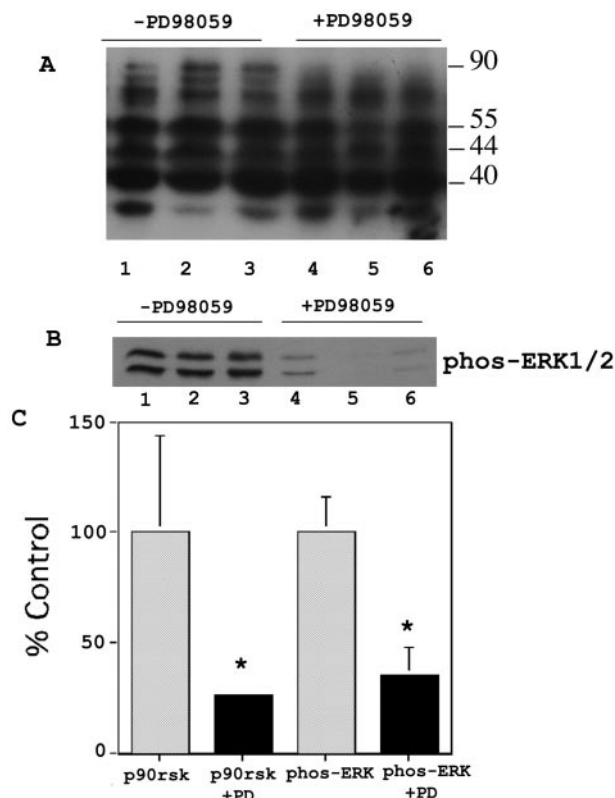


FIG. 8. In-gel kinase assay and Western blot analysis of isolated perfused rat hearts subjected to ischemia reperfusion in the presence or absence of 40  $\mu$ M of the MEK1 inhibitor PD98059. **A**, samples of ischemia-reperfused heart extracts were used in in-gel kinase analysis with the Na<sup>+</sup>/H<sup>+</sup> exchanger protein as a substrate. Lanes 1–3 are ischemic-reperfused hearts without PD98059; lanes 4–6 are ischemic-reperfused hearts treated with PD98059. **B**, Western blot analysis with anti-phospho-ERK1 antibody indicating the phosphorylated forms of ERK1/2. **C**, bar graph summarizing the in-gel kinase assay data and Western analysis data of five experiments. Asterisk (\*) indicates significant difference from untreated values at  $p < 0.5$ .

Na<sup>+</sup>/H<sup>+</sup> exchanger activity beyond the control levels. Nevertheless, it is clear that ERK-dependent phosphorylation was important in NHE1 activity and is important in resting p*H*<sub>i</sub>.

We also found that ischemia followed by reperfusion caused a large, transient, inhibitory effect on Na<sup>+</sup>/H<sup>+</sup> exchanger activity. The rate of recovery from an acid load was decreased as was the resting p*H*<sub>i</sub> (Fig. 2). This was unlikely to be due to nonspecific or proteolytic effects since it occurred reproducibly between 12 and 17 min after reperfusion, and then later was reversed. These results leave open the possibility that protein kinase mediated activation of the Na<sup>+</sup>/H<sup>+</sup> exchanger through ischemia reperfusion, may have an inhibitory effect on Na<sup>+</sup>/H<sup>+</sup> exchanger activity. Protein kinase-mediated inhibition of the Na<sup>+</sup>/H<sup>+</sup> exchanger has been suggested earlier in the heart (35) and smooth muscle (36). While it is not yet certain whether the physiological effects we observed are protein kinase mediated, they nevertheless demonstrate that ischemia followed by reperfusion has a significant physiological effect on Na<sup>+</sup>/H<sup>+</sup> exchanger activity.

We found that the p38 kinase was not a major regulator of Na<sup>+</sup>/H<sup>+</sup> exchanger activity in isolated myocytes. The inhibitor SB202190 caused only a slight, variable effect on the rate of recovery from an acute acid load. In addition, there was only a minor effect on the rate of recovery from an acute acid load in one group of isolated myocytes treated with severe ischemia followed by reperfusion. Our results agree with others that have suggested that p38 does not have significant activity toward the antiporter in the myocardium (37). However, they

disagree with those that suggest that p38 activity is important in smooth muscle (36). This difference could reflect dissimilar regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger which is known to be dependent on cell type (3, 38). Different isoforms of p38 could be responsible for the differential regulation of activity in different tissues (34).

To elucidate the specific kinases involved in the regulatory effects we examined the protein kinases that phosphorylated the antiporter during ischemia and reperfusion. Our initial experiments clearly demonstrate that both ischemia and ischemia reperfusion activate a number of protein kinases that phosphorylate the carboxyl-terminal region of the Na<sup>+</sup>/H<sup>+</sup> exchanger (Figs. 3–5). Similar to previous results (10), there were four major protein kinases that phosphorylated the antiporter. We found that ischemia reperfusion induced large increases (over 10-fold in some cases) in the activity of these kinases directed toward the Na<sup>+</sup>/H<sup>+</sup> exchanger. We used three models of ischemia reperfusion, the isolated perfused heart and two models of ischemia-treated isolated myocytes. One treatment was with cyanide alone to inhibit oxidative phosphorylation; the other more severe treatment also contained 2-deoxyglucose to also inhibit glycolysis. Ischemia alone increased most kinases' activity in the less severe ischemia model while in the more severe model, ischemia alone only increased phosphorylation by the 90-kDa protein. In contrast, severe ischemia, followed by reperfusion, increased most protein kinase activity toward the Na<sup>+</sup>/H<sup>+</sup> exchanger more than the less severe ischemia reperfusion. The reason for these differences is not known but it can be hypothesized that severe ischemia results in more ATP depletion that reduces protein kinase activity. Greater activation of the kinases by the severe ischemia was similar to the results obtained with the intact hearts, where ischemia and reperfusion caused up to 8-fold increases in kinase activity. These very large increases were surprising and extremely interesting observations. They suggest that that in the clinical situation of ischemia and reperfusion, Na<sup>+</sup>/H<sup>+</sup> exchanger-directed protein kinases may be greatly activated.

We attempted to identify the different protein kinases phosphorylating the Na<sup>+</sup>/H<sup>+</sup> exchanger. Immunoprecipitation experiments with anti-p90<sup>rsk</sup> and anti-ERK antibodies confirm the identity of these Na<sup>+</sup>/H<sup>+</sup> exchanger-directed protein kinases (10). In addition, they demonstrate that ischemia and reperfusion (Fig. 5) activate p90<sup>rsk</sup> and ERK-dependent phosphorylation of the Na<sup>+</sup>/H<sup>+</sup> exchanger. Our laboratory (9, 10) and others (5, 28, 36, 39, 40) have shown that MAP kinase-dependent pathways are important in regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger, although the details of the specific pathway involved appear to vary from one tissue to another. While one report (40) has suggested that ERK does not directly phosphorylate the Na<sup>+</sup>/H<sup>+</sup> exchanger, we have earlier shown that ERK directly phosphorylates the protein in stoichiometric amounts (9). In addition, we (10) and others (36) have more recently demonstrated ERK-mediated phosphorylation of the Na<sup>+</sup>/H<sup>+</sup> exchanger in muscle tissue. The reason for the discrepancy between some of these studies (40) is not known at this time but for now, the preponderance of evidence suggests that ERK directly phosphorylates the Na<sup>+</sup>/H<sup>+</sup> exchanger and is critical for regulation of activity (9).

The identification of the 90-kDa kinase as p90<sup>rsk</sup> is similar to a report in smooth muscle (41). The identification of the 44-kDa kinase as ERK1/2 is also similar to other reports in assays of myocytes and other tissues (5, 9, 28). However, there was an important discrepancy between the amount of phosphorylation observed in in-gel kinase assays of the 44-kDa protein and the amount of phospho-ERK detected in some samples. With isolated myocytes, the amount of 44-kDa kinase phosphorylating



the  $\text{Na}^+/\text{H}^+$  exchanger was increased by ischemia (Fig. 3A), however, anti-phospho ERK antibodies detected a decrease in phospho-ERK1 and -ERK2 (Fig. 3C, lanes 4–6). Similar results occurred with isolated perfused hearts (Fig. 5C) when ischemia increased the amount of phosphorylation by 44-kDa kinase but the amount of phospho-ERK was reduced. The most likely explanation is that there is another protein kinase at the same molecular weight as the ERK isoforms and that this kinase accounts for a large part of signal we see at this molecular weight. Immunoprecipitation showed that ERK1 is at least partly responsible for the phosphorylation observed at this molecular weight (Fig. 6E). However, after treatment of hearts with PD98059 there was an almost total absence of phosphorylated ERK1 and ERK2 protein (Fig. 8B), but no significant decrease in the level of 44-kDa protein kinase directed toward the  $\text{Na}^+/\text{H}^+$  exchanger. These results suggest that there is second protein kinase of ~44 kDa that phosphorylates the  $\text{Na}^+/\text{H}^+$  exchanger. It is likely a kinase in a pathway that is independent of the MAP kinase pathway since its activity remained in the presence of PD98059. In addition, its activity is stimulated by ischemia and reperfusion in both isolated myocytes and in the perfused intact heart.

It is known that many MAP kinase-dependent pathways are activated in the heart during ischemia. This includes the ERK kinases, p38 MAPK, and JNK kinases (22–26). Treatment of isolated perfused hearts increased the level of 44-kDa protein kinase that phosphorylated the carboxyl-terminal of the  $\text{Na}^+/\text{H}^+$  exchanger. While it is clear that this is not all due to ERK, immunoprecipitation experiments demonstrated that ERK1 was more active in tissues from hearts subjected to ischemia and reperfusion (Fig. 6E). Experiments with anti-ERK2 antibodies suggest that ERK2 was not phosphorylating the  $\text{Na}^+/\text{H}^+$  exchanger COOH terminus as strongly as ERK1 (not shown). The stimulation of ERK by ischemia and reperfusion is in agreement with previous studies (22, 22, 27, 28). These results demonstrated that ischemia and reperfusion activate ERK1 activity toward the  $\text{Na}^+/\text{H}^+$  exchanger.

$\text{p90}^{\text{rsk}}$  is a protein kinase downstream of ERKs. Similar to results with ERKs, it was possible to immunoprecipitate  $\text{p90}^{\text{rsk}}$  and demonstrate that the immunoprecipitated protein could phosphorylate the carboxyl-terminal of the  $\text{Na}^+/\text{H}^+$  exchanger (Fig. 6A). Both ischemia and ischemia reperfusion increased the level of phosphorylation by this protein kinase. Our results are in agreement with our earlier study (10) and that of others (37, 41) which suggest that  $\text{p90}^{\text{rsk}}$  is an important regulator of the  $\text{Na}^+/\text{H}^+$  exchanger. Hypoxia and reoxygenation have been shown to activate MAP kinase-dependent pathways earlier, including activation of  $\text{p90}^{\text{rsk}}$  (42). Our results demonstrate that ischemia and reperfusion of isolated hearts and isolated myocytes also increase  $\text{p90}^{\text{rsk}}$  activity toward the  $\text{Na}^+/\text{H}^+$  exchanger. Because of the significant role this kinase is known to play in  $\text{Na}^+/\text{H}^+$  exchanger regulation (10, 41), it is clear that this may be an important event in the activity of the antiporter during ischemia and reperfusion. It has recently been shown that reactive oxygen species can activate  $\text{p90}^{\text{rsk}}$  in pathways that are both MEK1/2-dependent and MEK1/2-independent (28, 43, 44). We found that PD98059 blocked ~60% of  $\text{p90}^{\text{rsk}}$  activity toward the  $\text{Na}^+/\text{H}^+$  exchanger (Fig. 8A), suggesting that both MEK1/2-dependent and MEK1/2-independent pathways could be involved in the present series of experiments.

Aside from the 44- and 90-kDa protein kinases that phosphorylate the  $\text{Na}^+/\text{H}^+$  exchanger, in-gel kinase assays demonstrated that two other protein kinases of 40 and 55 kDa also phosphorylated the  $\text{Na}^+/\text{H}^+$  exchanger. Both were highly stimulated markedly by ischemia and reperfusion. The identity of these protein kinases is not yet certain, however, their size and

activation by ischemia suggest that they could be p38, JNK (stress kinases), MEK1/2 (23–26), or CaM kinase II. Immunoprecipitation of p38 kinase showed that this kinase might not account for the 40-kDa  $\text{Na}^+/\text{H}^+$  exchanger-directed protein kinase. It should be noted, however, that immunoprecipitation of p38 from other cells types led to strong phosphorylation of the  $\text{Na}^+/\text{H}^+$  exchanger in IGKAs (not shown). It may be that the isoform of p38 present in the myocardium was not as reactive toward the  $\text{Na}^+/\text{H}^+$  exchanger as in other tissues, or that its abundance and specificity was below the level of detection of our IGKA. Future experiments will examine this in more detail. At present our IGKA results largely agree with our physiological experiments that have shown that the p38 pathway plays a minor role in activity of the  $\text{Na}^+/\text{H}^+$  exchanger in the myocardium.

The identity of the 55-kDa protein kinase is not known at this time. Immunoprecipitation of JNK2 and an examination of its activity in in-gel kinase assays suggested that it likely does not account for this  $\text{Na}^+/\text{H}^+$  exchanger-directed kinase we see in our assays. A similar result was found for CaM kinase II and MEK. The lack of activity of JNK toward the  $\text{Na}^+/\text{H}^+$  exchanger agrees with the results of Kusuhara *et al.* (36) in smooth muscle. It should be noted, however, that it is possible that JNK1 phosphorylates the  $\text{Na}^+/\text{H}^+$  exchanger since our immunoprecipitates only contain the JNK2 isoform of the protein. Further studies will be necessary to identify the 55-kDa protein kinase in the myocardium.

Regulation of the  $\text{Na}^+/\text{H}^+$  exchanger is a balance between phosphorylation of the protein and dephosphorylation of the many potential sites available on the carboxyl-terminal region. We found that ischemia and reperfusion resulted in a transient decrease in the activity of the protein. Recently, two reports have suggested that protein kinase-mediated phosphorylation can be inhibitory to the  $\text{Na}^+/\text{H}^+$  exchanger (35, 36). It is well known that the activity of the  $\text{Na}^+/\text{H}^+$  exchanger is detrimental to the myocardium during ischemia and reperfusion (12, 13). It is therefore tempting to suggest that a protein kinase-mediated inhibitory mechanism exists for reducing  $\text{Na}^+/\text{H}^+$  exchanger activity during reperfusion. Such a mechanism could help reduce the damage the  $\text{Na}^+/\text{H}^+$  exchanger activity normally mediates (12, 13). Our study demonstrates that several unknown protein kinases of size 55, 44, and 40 kDa are activated during ischemia followed by reperfusion. Further experiments are necessary to examine if these kinases mediate the inhibitory effects we found on the  $\text{Na}^+/\text{H}^+$  exchanger.

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