The activity of the Na\(^+\)/H\(^+\) 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\(^+\)/H\(^+\) exchanger. The Na\(^+\)/H\(^+\) 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\(^{rsk}\) 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\(^+\)/H\(^+\) exchanger. The results demonstrate that MAPK-dependent pathways including p90\(^{rsk}\) and ERK1/2 and are important in regulating the Na\(^+\)/H\(^+\) exchanger and show their dramatic increase in activity toward the Na\(^+\)/H\(^+\) exchanger during ischemia and reperfusion of the myocardium. They also show that ischemia followed by reperfusion have important inhibitory effects on Na\(^+\)/H\(^+\) exchanger activity.

The Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1)\(^1\) 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\(_i\)) in most mammalian cells (1, 2). Of the six known isoforms of the Na\(^+\)/H\(^+\) 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\(^{rsk}\) (5, 6). However, earlier work has demonstrated growth factor induced phosphorylation of several different peptides of the carboxyl-terminal region of the Na\(^+\)/H\(^+\) 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\(^{rsk}\) are involved in regulation of exchanger activity in the healthy rat myocardium (10). Another protein kinase, Ca\(^2+\)/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\(^+\)/H\(^+\) exchanger during ischemia and reperfusion produces numerous secondary effects that lead to the exacerbation of tissue injury. The Na\(^+\)/H\(^+\) exchanger removes protons either during ischemia or during reperfusion which causes excess intracellular Na\(^+\). This results in either inhibition of the Na\(^+\)/Ca\(^2+\) exchangers ability to extrude Ca\(^2+\), or reversal in activity of the Na\(^+\)/Ca\(^2+\) exchanger and accumulation of Ca\(^2+\). The increased intracellular Ca\(^2+\) results in a variety of detrimental effects to the heart. It has been shown that inhibition of Na\(^+\)/H\(^+\) 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\(^+\)/H\(^+\) exchanger may be in an activated state during ischemia and/or reperfusion of the myocardium. α1-Adrenergic receptor stimulation regulates the antiporter in the myocardium causing both an alkalization of steady state intracellular pH (pH\(_i\)) and an enhanced rate of Na\(^+\)/H\(^+\) exchanger mediated recovery from an acid load (14–16). α1-Adrenergic stimulation is known to exacerbate reperfusion induced arrhythmias and increased Na\(^+\)/H\(^+\) 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\(^+\)/H\(^+\) exchange in cardiac myocytes (18, 19). Under some circumstances endothelin may also aggravate ischemic reperfusion injury, possibly through Na\(^+\)/H\(^+\) 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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1 The abbreviations used are: NHE1, Na\(^+\)/H\(^+\) 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\(_2\)-terminal kinase 1 and 2; GST, glutathione S-transferase; MAPK, mitogen activated protein kinase; MEK1/2, MAPK kinase 1 and 2; pH\(_i\), intracellular pH; IGKA, in-gel kinase assay; KRH, Krebs-Ringer-HEPES; PAGE, polyacrylamide gel electrophoresis.

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ACTIVATION OF \( \text{Na}^+ / \text{H}^+ \) EXCHANGER-DIRECTED PROTEIN KINASES IN THE ISCHEMIC AND ISCHEMIA-REFUSING RAT MYOCARDIUM*
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), p38MAPK, and c-Jun NH2-terminal kinases (JNKs) are activated in ischemic-reperfusion 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 (H2O2) show increased Na+/H+ 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+/H+ 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+/H+ exchanger. The purpose of our study was to examine regulation of the Na+/H+ 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+/H+ exchanger. This present study provides the first evidence of protein kinase-mediated regulation of the Na+/H+ exchanger in response to ischemia and ischemia reperfusion. The results also suggest that regulation of the myocardial Na+/H+ 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 4B 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-RSK (p90 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 (Santa Cruz, CA). Anti-ERK-1/2 (rabbit polyclonal), anti-p38, anti-RSK1 (goat polyclonal), anti-CaM kinase II (goat polyclonal), anti-JNK2 (rabbit polyclonal), and anti-MEK1 (rabbit polyclonal) were from Santa Cruz Biotechnology (Santa Cruz, CA). 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 (Santa Cruz, CA). 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 (Santa Cruz, CA). 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 (Santa Cruz, CA). 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 (Santa Cruz, CA). 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 (Santa Cruz, CA). 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 (Santa Cruz, CA).

In-gel Kinase Assays—To identify protein kinases that phosphorylated the Na+/H+ exchanger fusion protein, cell extracts (80 μg of protein) from control, ischemic, and ischemia-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 rsk1, p38, JNK2, and MEK1 from Control, Ischemic, and Ischemic-reperfused Heart Cells—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-phospho-ERK, and 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 resin. 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 A-Sepharose CL-4B beads were used, and the elution buffer was 100 mM glycine–HCl (pH 2.5). Protein A-Sepharose CL-4B was obtained from Sigma, or BDH (Toronto, ON). Construction and Purification of Glutathione S-Transferase-Na+/H+ Exchanger Fusion Protein—The carboxyl-terminal 178 amino acids of the rabbit cardiac Na+/H+ exchanger were expressed as described previously (9) as a fusion protein with GST (PCRA) using the plasmid pGEX-3X. The Escherichia coli TOTP 2 strain was induced with 1 mM isopropylthio-β-d-galactoside. GST-Na+/H+ 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 Harlan Sprague-Dawley rat heart ventricles as described previously (10). Isolated primary myocytes were plated onto glass coverslips for physiologic studies, or onto Primaria® (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/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 μM pyruvic acid, 0.1 mM minimum essential medium non-essential amino acids, 10% MEM vitamin, 0.1 mM bromodeoxyuridine, 100 μM t-asorbic 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, 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 I. 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 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 MP-20 (cardiac sarcomeric myosin heavy chain) and for their ability to phosphorylate the Na+/H+ 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 acetyloxyethyl ester of 2‘-7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein was used to measure steady-state pH, as described previously (9, 32). Briefly, pH, was measured using the dual excitation single emission ratio technique using a temperature-controlled Shimadzu RF5000 spectrofluorimeter. Excitation wavelengths were at 452 and 503 nm with emission at 524 nm. Myocytes were serum-starved before all pH measurements. Cells on coverslips were placed into a holder device and inserted into a microscope. The tube connection was 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 MP-20 (cardiac sarcomeric myosin heavy chain) and for their ability to phosphorylate the Na+/H+ exchanger.

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 (I) 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+-free buffer. For measuring initial rate of recovery from an acid load we examined the initial changes in pH 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 loads divided by the observed change of cell pH produced by this load. Buffering capacity was determined at various pH, by varying amounts of ammonium chloride. Proton flux (DpH/t) produced due to the Na+/H+ exchanger after acid loading was calculated from the buffer capacity at mid point pH × 3pH/A time essentially as described earlier (33).

Data Analysis—Autoradiographs were quantified using a model BAS2500 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, 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.
NHE1 Protein Kinases in Myocardial Ischemia Reperfusion

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 μM PD98059 or 1 μM SB202190. Ischemia was induced by the presence of KCN (2.5 mM) or KCN plus 2-deoxy-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 lanes used for pH recovery measurements. A, illustrates representative tracings of pH$_i$ recovery of myocytes. B and C, bar graphs summarize the results of pH$_i$ recovery from an acute acid load. Results are mean ± 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. δ indicates significant difference from no SB202190 treatment at p < 0.01.

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. 2, 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 ~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

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).
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 Na+/H+ Exchanger—To more conclusively identify ERK1/2 and p90<sup>rsk</sup> (RSK1) as activated protein kinases that phosphorylate the carboxyl-terminal of the Na+/H+ exchanger, these kinases were immunoprecipitated with antibodies specific to ERK1/2 and p90<sup>rsk</sup> (RSK1). Specific kinases were immunoprecipitated from cell extracts of control (Fig. 6, lanes 1–3), ischemia (lanes 4–6), and ischemic-reperfusion (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<sup>rsk</sup> was activated and phosphorylated the Na+/H+ exchanger more than controls. p90<sup>rsk</sup> was highly activated during ischemia and ischemic reperfusion in comparison to either control hearts or hearts treated with ischemia alone. A similar result occurred with...
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. 6F). The in-gel kinase assay showed that ERK1 was highly activated during ischemia reperfusion and phosphorylated the Na+/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. 6C). 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 during ischemia followed by reperfusion. However, immunoprecipitation of ERK2 with anti-ERK2 antibodies suggested that ERK2 does not appear to phosphorylate the Na+/H+ exchanger as strongly as ERK1 (data not shown).

Activity of p38 and JNK2 in IGKAs Directed Toward the Na+/H+ Exchanger—To identify p38 (38-kDa) and/or JNK2 (54-kDa) (stress kinases) as activated protein kinases that phosphorylate the Na+/H+ exchanger, these kinases were immunoprecipitated with specific antibodies. Antibody to JNK2 recognizes both JNK1/2 in Western analysis, but only immunoprecipitates JNK2 (54 kDa) (stress kinases) as activated protein kinases that phosphorylate the Na+/H+ exchanger. Samples of immunoprecipitates and cell extracts before and after immunoprecipitation were then used for in-gel kinase assays with Na+/H+ exchanger 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 (IR) 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 rsk (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.
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 Na⁺/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 Na⁺/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 ATP-2 substrate.

It is well established that the activity of the Na⁺/H⁺ exchanger exacerbates the tissue injury that occurs during ischemia and reperfusion of the myocardium (4, 12). However, regulation of the activity of the Na⁺/H⁺ exchanger during this time period has not been studied. To investigate this phenomenon further, we examined protein kinase-mediated regulation of the Na⁺/H⁺ exchanger during ischemia and reperfusion of the myocardium. We initially demonstrate that ERK-dependent pathways are involved in activity of the Na⁺/H⁺ exchanger when recovering from ischemia and reperfusion. When isolated myocytes were exposed to ischemia followed by reperfusion, PD98059 also inhibited the ability to recover from an acute acid load. Because of the time required for inducing acute acid load, it was not possible to measure the effects of reperfusion on Na⁺/H⁺ exchanger very early after ischemia. Therefore it was not possible to examine if very early reperfusion elevates

cipated p38 from heart extracts. Similarly, Fig. 7D shows no phosphorylation of the Na⁺/H⁺ exchanger by immunoprecipitated JNK2. The results demonstrated that neither our immunoprecipitate of p38 nor that of JNK2, phosphorylate the carboxyl-terminal of the Na⁺/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 Na⁺/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 Na⁺/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 μM SB202190. There was no reduction of any specific molecular weight protein kinase activity directed toward the Na⁺/H⁺ exchanger, despite a general overall reduction in all protein kinases activity (not shown).

**Inhibition of MEK1 by PD98059 Reduces Na⁺/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 μM PD98059, a MEK1 inhibitor. The use of PD98059 reduced the phosphorylation activity of the 90-kDa kinases toward the Na⁺/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 Na⁺/H⁺ exchanger exacerbates the tissue injury that occurs during ischemia and reperfusion of the myocardium (4, 12). However, regulation of the activity of the Na⁺/H⁺ exchanger during this time period has not been studied. To investigate this phenomenon further, we examined protein kinase-mediated regulation of the Na⁺/H⁺ exchanger during ischemia and reperfusion of the myocardium. We initially demonstrate that ERK-dependent pathways are involved in activity of the Na⁺/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 Na⁺/H⁺ exchanger to recover from an acute acid load. Because of the time required for inducing acute acid load, it was not possible to measure the effects of reperfusion on Na⁺/H⁺ exchanger very early after ischemia. Therefore it was not possible to examine if very early reperfusion elevates
was the resting pH activity. The rate of recovery from an acid load was decreased as Western blot analysis with anti-phospho-ERK1 antibody indicating the lanes 4–6 are ischemic-reperfused hearts treated with PD98059. (* ) indicates significant difference from untreated values at kinase assay data and Western analysis data of five experiments. Lanes 1–3 substrate.

reperfusion has a significant physiological effect on Na+/H+ exchanger activity. While it is not yet certain whether the Na+/H+ exchanger has been suggested earlier in the heart (35) and smooth muscle (36). However, they 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+/H+ 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+/H+ 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+/H+ exchanger. We used three models of ischemia reperfusion, the isolated perfused heart and two models of ischemia-treated isolated myocytes. One treatment was with sodium 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+/H+ 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 in the clinical situation of ischemia and reperfusion, Na+/H+ exchanger-directed protein kinases may be greatly activated.

We attempted to identify the different protein kinases phosphorylating the Na+/H+ exchanger. Immunoprecipitation experiments with anti-p90 

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 μM of the MEK1 inhibitor PD98059. A, samples of ischemia-reperfused heart extracts were used in in-gel kinase analysis with the Na+/H+ 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.
the Na"/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 phospho-ERK1 and ERK2 protein (Fig. 8F), but no significant decrease in the level of 44-kDa protein kinase directed toward the Na"/H" exchanger. These results suggest that there is second protein kinase of ~44 kDa that phosphorylates the Na"/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 Na"/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 Na"/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 Na"/H" exchanger.

p90"rsk is a protein kinase downstream of ERKs. Similar to results with ERKs, it was possible to immunoprecipitate p90"rsk and demonstrate that the immunoprecipitated protein could phosphorylate the carboxyl-terminal of the Na"/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 p90"rsk is an important regulator of the Na"/H" exchanger. Hypoxia and reoxygenation have been shown to activate MAP kinase-dependent pathways earlier, including activation of p90"rsk (42). Our results demonstrate that ischemia and reperfusion of isolated hearts and isolated myocytes also increase p90"rsk activity toward the Na"/H" exchanger. Because of the significant role this kinase is known to play in Na"/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 p90"rsk in pathways that are both MEK1/2-dependent and MEK1/2-independent (28, 43, 44). We found that PD98059 blocked ~60% of p90"rsk activity toward the Na"/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 Na"/H" exchanger, in-gel kinase assays demonstrated that two other protein kinases of 40 and 55 kDa also phosphorylated the Na"/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 Na"/H" exchanger-directed protein kinase. It should be noted, however, that immunoprecipitation of p38 from other cell types led to strong phosphorylation of the Na"/H" exchanger in IGKAs (not shown). It may be that the isoform of p38 present in the myocardium was not as reactive toward the Na"/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 Na"/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 Na"/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 Na"/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 Na"/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 Na"/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 Na"/H" exchanger (35, 36). It is well known that the activity of the Na"/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 Na"/H" exchanger activity during reperfusion. Such a mechanism could help reduce the damage the Na"/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 Na"/H" exchanger.

REFERENCES
