Protein Phosphatase Regulation of Na^+/H^+ Exchanger Isoform I†

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ABSTRACT: We investigated regulation of Na^+/H^+ exchanger isoform 1 (NHE1) by dephosphorylation. Treatment of primary cultures of cardiomyocytes with the phosphatase inhibitor okadaic acid increased the rate of recovery from an acid load, suggesting that the okadaic acid sensitive PP1 may be involved in NHE1 regulation in vivo. We examined the ability of purified protein phosphatases PP1, PP2A, and PP2B to dephosphorylate the regulatory cytoplasmic tail. NHE1 was completely dephosphorylated by PP1, poorly dephosphorylated by PP2A, and not dephosphorylated by PP2B. Examination of NHE1 binding to PP1 or PP2B revealed that an association occurs between NHE1 and PP1 both in vitro and in vivo, but NHE1 did not associate with full-length PP2B. We expressed PP1 or inhibitor 2, a specific PP1 inhibitor, in cell lines to examine the effect of PP1 on NHE1 activity in vivo. Overexpression of PP1 causes a decrease in NHE1 activity but does not affect stimulation by thrombin. Cell lines expressing the specific PP1 inhibitor, inhibitor 2, had elevated proton efflux rates and could not be further stimulated by the Na^+/H^+ exchanger agonist thrombin. The results suggest that PP1 is an important regulatory phosphatase of NHE1, that it can bind to and dephosphorylate the protein, and that it regulates NHE1 activity in vivo.

Mammalian Na^+/H^+ exchanger isoform 1 (NHE1)[1] is a ubiquitously expressed integral membrane protein that mediates the exchange of one extracellular sodium ion for one intracellular proton (*1*). It protects cells from intracellular acidification (*2*, *3*), stimulates changes in the growth or functional state of cells (*2*), and regulates both sodium fluxes and cell volume after osmotic shrinkage (*4*). The Na^+/H^+ exchanger plays an important role in the damage that occurs to the human myocardium during ischemia and reperfusion, and inhibition of the exchanger protects the myocardium during these events (*5*). Activation of the protein by phosphorylation occurs in the myocardium during ischemia and acidosis, which may aggravate the damage that occurs during heart disease (*6*, *7*).

The Na^+/H^+ exchanger is composed of two domains: the N-terminal membrane domain of approximately 500 amino acids and a C-terminal regulatory domain approximately 300 amino acids in length (*1*, *5*). The cytoplasmic domain is involved in mediating regulation of the membrane domain by pH*i*, by cytosolic calcium (Ca^{2+}) binding proteins, by cellular ATP, and by protein phosphorylation (*8*). Removal of the entire NHE1 cytosolic tail causes an acidic shift of pH*i* dependence of exchange activity and the disappearance of growth factor-induced cytoplasmic alkalinization (*9*).

Stimulation of the Na^+/H^+ exchanger by mitogens and phorbol esters is associated with the activation of protein kinases and enhanced phosphorylation of the exchanger (*10*−*13*). Phosphorylation occurs in the distal 178 amino acids of the cytoplasmic domain (*12*, *14*) and accounts for approximately 50% of mitogen-induced NHE1 activation (*14*). The phosphorylation of NHE1 leads to a shift in the pH*i* sensitivity of the modifier site, which accounts for the activation of exchange activity and cytosolic alkalinization (*9*, *10*). The current list of putative NHE1 kinases includes Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (*15*), extracellular signal-regulated kinase (ERK) (*12*), p160ROCK (*16*), the 90 kDa ribosomal S6 kinase ($p90^{RSK}$) (*17*), p38 mitogen-activated protein kinase (p38 MAPK) (*18*), and Nck-interacting kinase (NIK) (*19*). The exact amino acids phosphorylated by all the protein kinases are not certain, but studies have shown that amino acids Ser 703, Thr 717, Ser 722, Ser 725, and Ser 728 are phosphorylation targets (*17*, *18*).

While there have been numerous studies attempting to elucidate the kinases involved in activation of the Na^+/H^+ exchanger, there have been few examining the requisite dephosphorylation of the protein that must occur after activation. Most of these have used pharmacological approaches to gain a basic idea of which protein phosphatases could be involved in regulating activity. In this regard, okadaic acid is a polyether derivative of a fatty acid that has different efficacies toward the various phosphatase isoforms (*20*). It is a potent inhibitor of PP2A, has intermediate efficacy toward PP1 (100-fold less active), and is a poor

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[1] Abbreviations: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester; CaM, calmodulin; CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; HA, hemagglutinin; I2, inhibitor 2; NHE1, Na^+/H^+ exchanger isoform 1; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B.

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inhibitor of PP2B (*21*). In platelets, rat thymic lymphocytes, human bladder carcinoma cells, and Chinese hamster lung fibroblasts, okadaic acid can directly improve the phosphorylation and activity of the NHE (*10*, *13*, *22*, *23*). However, okadaic acid can also act indirectly to increase the activity of ERK1/2 and consequently activate NHE1 in intact human red blood cells (*12*, *24*). It is therefore difficult to be certain that activation of the Na^+/H^+ exchanger by pharmacological treatments in vivo is due to an effect on a specific phosphatase that directly dephosphorylates the Na^+/H^+ exchanger.

Few studies have examined the protein phosphatases that directly regulate NHE1. On the basis of the differential sensitivity to phosphatase inhibitors, it has been suggested that the trout βNHE protein is regulated by phosphatase PP1 (*25*−*27*). However, direct evidence of the role of this phosphatase in regulating the mammalian NHE1 isoform of the Na^+/H^+ exchanger is lacking. In this study, we examine which of the PPP family (*28*) of protein phosphatases, PP1, PP2A, and PP2B (*29*), are responsible for dephosphorylation of the NHE1 isoform of the Na^+/H^+ exchanger. Our results show that PP1 can bind to and regulate the activity of the Na^+/H^+ exchanger in vivo. Our study is the first examination of the direct effects of PP1 in regulation of the Na^+/H^+ exchanger.

EXPERIMENTAL PROCEDURES

Materials. A plasmid containing the catalytic subunit of PP1 (pCW-HPP1γ) has been described previously (*30*). To obtain the cDNA fragment of human PP1 inhibitor 2 containing the coding sequence for inhibitor 2, a PCR amplification using Human Teratocarcinoma library (Stratagene) as a template was performed. An NdeI restriction site was engineered at the initiating methionine codon with PCR. Primer 5′-GCGCCATATGGCGGCCTCGACGGCCTCGCACCGGCCCATCAAGGGG-3′ was used for the 5′ end of the coding sequence, and primer 5′-ACTTTGTAAGAGCTACCACATTTCAAGTGATGAAAATAAATTAGTTCCCCCCC-3′ was used for the 3′ end. The PCR was carried out using platinum Tag high-fidelity DNA polymerase (Invitrogen) in all 100 reactions. Conditions were 94 °C for 1 min, −58 °C for 1 min, and −68 °C for 3 min for 35 cycles. The fidelity of the 1 kb PCR product was checked by DNA sequencing to confirm the coding sequence of inhibitor 2. The PCR product was inserted into the expression vector PET3a, and both were digested with NdeI and ClaI. E. Olson (The University of Texas Southwestern Medical Center, Dallas, TX) provided the plasmid (pcDL-SRα296) used as a source of constitutively active PP2B (*31*). The Gateway Cloning and expression vectors (pDONR201, pDEST40), all PCR reagents, cell culture media, supplements, and Opti-MEM transfect ion media were purchased from Invitrogen Life Technologies (Carlsbad, CA). Restriction enzymes were purchased from Invitrogen Life Technologies or New England Biolabs (Beverly, MA). MWG Biotech, Inc. (High Point, NC) and Qiagen (Mississauga, ON) supplied oligonucleotides for DNA sequencing and PCR. Topoisomerase I was purchased from Invitrogen Life Technologies. The fluorescent probe 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR). Other commercial kits were the Bio-Rad (Hercules, CA) DC protein assay kit for protein quantification and the QiaQuick (Qiagen) PCR purification kit for purification of PCR products.

AP1 cells were a generous gift from S. Grinstein (University of Toronto, Toronto, ON). CHO cells were purchased from American Type Culture Collection. AP1-NHE1 cells are AP1 cells overexpressing the human NHE1 isoform of the Na^+/H^+ exchanger and have been described previously (*32*). The cell culture media αMEM, thrombin, and nigericin were purchased from Sigma Aldrich (Oakville, ON). The transfection reagent Lipofectamine 2000 was purchased from Invitrogen Life Technologies. DSP, dithiobis(succinimidyl-propionate), was purchased from Pierce Biotechnology (Brockville, ON).

Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (anti-PP1 polyclonal antibody and anti-PP2B-A polyclonal antibody), Invitrogen Life Technologies (anti-V5 epitope antibody), and Chemicon International, Inc. (Temecula, CA) (anti-NHE1 monoclonal antibody). The anti-PP1 monoclonal antibody was a kind gift from M. Pato (University of Saskatchewan, Saskatoon, SK), and the anti-NHE1 polyclonal antibody was developed previously (*33*).

Construction and Purification of Proteins. The C-terminal 178-amino acid sequence of the Na^+/H^+ exchanger was expressed as a fusion protein with glutathione *S*-transferase (GST) (NHEC-178) using the plasmid pGEX-3X as described previously (*34*). The *Escherichia coli* TOPP2 strain was induced with 1 mM isopropyl thio-D-galactoside. NHEC-178 was purified via glutathione−Sepharose 4B affinity chromatography as described previously (*34*).

Protein phosphatases were expressed and purified for in vitro dephosphorylation of the Na^+/H^+ exchanger. The catalytic subunit of the human PP1γ was expressed in *E. coli* strain DH5α using the plasmid pCW and subsequently purified using heparin affinity, Mono Q, and Superdex-75 gel filtration chromatography as previously described (*35*−*37*) with the following modifications. A single colony of DH5α *E. coli* cells transformed with the plasmid pCW-HPP1γ was used to inoculate 100 mL of LB medium containing 1 mM $MnCl_2$ and 54 μM ampicillin. After overnight growth at 37 °C, this culture was used to inoculate 1 L of LB medium containing 1 mM $MnCl_2$ and 54 μM ampicillin and was grown to an optical density of 0.5 at 600 nm. Expression was then induced with 1 mM IPTG for up to 18 h. Cells were harvested by centrifugation for 45 min at 4000*g*, and either frozen at −70 °C until use or used immediately. Bacterial cells from two 1 L cultures were resuspended in 80 mL of buffer A [50 mM imidazole (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM $MnCl_2$, 3 mM DTT, 2 mM benzamidine, 0.5 mM PMSF, and 10% (v/v) glycerol] and disrupted by sonication. The lysate was then centrifuged at 16000*g* for 1 h at 4 °C. The supernatant was then loaded onto an 80 mL heparin−Sepharose column (Pharmacia) equilibrated in buffer A. The column was run with a 400 mL linear gradient from 0.1 to 0.5 M sodium chloride, and 5 mL fractions were collected at a flow rate of 5 mL/min. Fractions were assayed for activity using *p*-nitrophenyl phosphate (pNPP) as a substrate. Ten microliters of the fraction was added to 50 μL of buffer containing 50 mM Tris (pH 8.3), 34 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM $MnCl_2$, 0.2% 2-mercaptoethanol, and 1 mg/mL BSA. Ten microliters of 30 mM pNPP was added to the

reaction mixture, and the reaction mixture was allowed to incubate at room temperature for 20 min. The absorbance of the individual reactions was then read at 280 nm. The active fractions were pooled and diluted 2:1 with buffer B [50 mM imidazole (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 3 mM $MnCl_2$, 3 mM DTT, 2 mM benzamidine, 0.5 mM PMSF, and 10% (v/v) glycerol] and loaded onto a MonoQ 10/10 column (Pharmacia). The column was run with a 160 mL linear gradient from 50 to 400 mM sodium chloride, and 2 mL fractions were collected at a flow rate of 2 mL/min. The active fractions were pooled and concentrated to a volume of 1.5−10 mL using Amicon ultracentrifugal filter devices (Millipore) and loaded onto a HiLoad 26/60 Superdex 75 prep grade column (Pharmacia) equilibrated in buffer C [50 mM imidazole (pH 7.5), 300 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM $MnCl_2$, 3 mM DTT, 2 mM benzamidine, and 10% (v/v) glycerol]. The column was run with a 180 mL isocratic gradient, and 0.6 mL fractions were collected for the final 54 mL at a flow rate of 0.2 mL/min. Active fractions were pooled and concentrated to a volume of 1−3 mL. Glycerol was added to a final concentration of 50%, and PP1 was then stored at −20 °C.

The catalytic A subunit of rat calcineurin was expressed in the *E. coli* HMS174(DE3) strain, using the pET-21a(+) vector, and subsequently purified as described previously (*38*) with the addition of the Toyopearl HW65 column used after calmodulin−Sepharose affinity and before using gel filtration chromatography. Calcineurin activity was routinely assayed to ensure enzyme activity as described previously (*39*). The catalytic subunit of PP2A was purified from bovine cardiac muscle as previously described (*40*) using a DEAE-Sepharose column, the poly(L-lysine)−Sepharose affinity column, the Superdex 75 column, and the Mono Q anion exchange column (*35*, *40*). Calmodulin was produced and purified as described previously (*41*).

In Vitro Phosphorylation and Dephosphorylation of the Na^+/H^+ Exchanger Fusion Protein. In vitro phosphorylation of NHEC-178 was performed as previously described (*12*). Control reaction mixtures did not contain heart extracts. The phosphorylated NHEC-178 fusion protein (12.5 μL) was immobilized using 10 μL of glutathione−Sepharose (Sigma) beads, and initially lightly shaken for 15 min at room temperature. The supernatant was removed, and the beads were washed three times with 1× PBS. Twenty-five microliters of the standard dephosphorylation reaction mix was added to the beads and the mixture incubated at room temperature with light shaking for 90 min. It contained 12.5 mM MOPS (pH 7.0), fresh 2 mM dithiothreitol, 8.5 mM magnesium chloride, 10 μg of purified phosphatase (PP1, PP2A, or PP2B), or 8.0 μg of heart extracts. In addition, reaction mixtures with PP1 contained 5 mM manganese chloride; reaction mixtures with PP2A were made ±5 mM manganese chloride, and reaction mixtures with PP2B contained 10 mM calcium chloride and 5 μg of purified calmodulin. The total volume of each reaction mixture was 25 μL. Control reaction mixtures did not contain phosphatases or divalent cations. PP1 and PP2A had comparable activity versus the substrate phosphorylase *a*. The reactions were terminated with SDS loading buffer. Samples were run on a 10% SDS−PAGE gel, dried, and exposed for autoradiography. For both the phosphorylation and dephosphorylation of NHE1, the ^{32}P-labeled protein bands observed on the autoradiogram and the protein bands stained with Coomassie blue were quantified using Image Gauge as described previously (*42*).

Preparation of Cell Extracts from Adult Rat Myocardium. Extracts were prepared from ventricles of untreated adult Harlan Sprague-Dawley rats as previously described (*12*). The homogenate was sonicated for 30 s at 4 °C prior to centrifugation at 6000*g* for 60 min at 4 °C.

Construction of Protein Phosphatase Expression Vectors. Plasmids were made to express phosphatases PP1 and PP2B and inhibitor 2 (I2) in mammalian cells. In all cases, the Gateway Cloning System (Invitrogen) was used with the pDONR201 and pDEST40 vectors. pDEST40 contains a CMV promoter, a V5 epitope, a growth hormone polyadenylation sequence, and a neomycin resistance gene allowing selection of stable cell lines. Forward primers contained a Shine-Dalgarno sequence and a Kozak consensus sequence to provide optimal conditions for translation of the gene. Reverse primers allowed for expression of the V5 epitope that was used to confirm expression of the protein. The templates for polymerase chain reaction are described above. The primers are listed in Table 1. PCR amplification and cloning were as described previously (*43*). The resultant plasmids are pDEST40-HPP1γ, pDEST40-I2, and pDEST40-Cn encoding human PP1γ, human I2, and constitutively active calcineurin, respectively.

Cell Culture and Transfections. A Chinese hamster ovary (CHO) cell line was grown in a humidified atmosphere of 5% CO_2 and 95% air in αMEM medium supplemented with 10% (v/v) fetal bovine serum, 25 mM HEPES, penicillin (100 units/mL), and streptomycin (100 μg/mL) at pH 7.4 and 37 °C. Transient transfections (24 h) and stable transfections were made using Lipofectamine 2000 (Life Technologies, Inc., Rockville, MD) essentially as described previously (*32*). Plasmids pDEST40-HPP1γ, pDEST40-I2, and pDEST40-Cn were used. For control experiments, CHO cells were mock transfected with an empty pDEST40 vector. All results with stable cell lines are typical of at least two independently made cell lines.

Five- to six-day-old Harlan Sprague-Dawley rats were used for generating neonatal primary culture cardiac myocytes. Isolated cardiomyocytes were prepared and maintained as described previously (*7*).

Measurement of Intracellular pH. NHE activity on coverslips was measured using BCECF as described previously (*7*, *32*). pH regulation by the Na^+/H^+ exchanger was examined in (mock-transfected) CHO cells and CHO/pDEST40-HPP1γ and pDEST40-I2 stably transfected cells. Cells on coverslips were incubated with BCECF-AM for 45 min at 37 °C, placed into a holding device, and inserted into a fluorescence cuvette at 37 °C. In some cases, the rate of recovery from an ammonium chloride-induced acid load (40 mM) was measured in Na^+-containing buffer in the presence and absence of thrombin (2 units/mL). For these experiments, after ammonium chloride-induced acidosis, thrombin (2 units/mL) was introduced into Na^+-containing buffer for 3 min. Following this incubation period, the rate of recovery from an acid load was measured in Na^+-containing buffer in the presence of thrombin. pH measurements were taken with a PTI Deltascan spectrofluorophotometer. To calculate proton efflux, the buffering capacity at varying pHs was determined. Buffering capacity B and proton efflux J_{H^+} were determined

Table 1: Synthetic Oligonucleotides Used for Cloning and Expression of Protein Phosphatases and Inhibitor 2

name	primer sequence[a]
PP1 forward primer	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACC**ATGGCGGATTTAGATAAACTCAACATCG** 3′
PP1 reverse primer	5′ GGGGACCACTTTGTACAAGAAAGCTGGGT**ATTTCTTTGCTTGCTTTGTGATCATACC** 3′
I2 forward primer	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACC**ATGGCGGCCTCGACGGCCTCGCACCGG** 3′
I2 reverse primer	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTT**TGAACTTCGTAATTTGTTTTGCTGTTGG** 3′
PP2B forward primer	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACC**ATGTCCGAGCCCAAGGCGATTGATCC** 3′
PP2B reverse primer	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTC**CTACTTGTTTCTGATGACTTCCTTC** 3′

[a] Lightface type represents *att*B (recombination) sequences, including SD and Kozak consensus sequences. Boldface type represents the sequence of the gene of interest.

as described previously (*44*). Proton flux produced due to the Na^+/H^+ exchanger after acid loading was estimated as the product of the rate of pH*i* change (dpH/d*t*) and buffering capacity (millimoles per liter per pH unit) as described previously (*44*).

$$\text{proton flux } (J_H^+) = \mathrm{d}(B \times \mathrm{pH})/\mathrm{d}t$$

For experiments using stable cell lines, rates of recovery are expressed as proton flux, to take into account any differences in buffering capacity. For acute treatment with agonists, the rate of recovery was used; the degree of ammonium chloride-induced acidification did not vary between treated and untreated cells. Statistical significance was determined using the Mann−Whitney U test.

GST Pull-Down Assays. GST pull-down experiments were performed using purified PP1 or PP2B and NHEC-178 to examine physical interactions between protein phosphatases PP1 and PP2B and the cytoplasmic tail of the Na^+/H^+ exchanger. Each experiment included 20 μg of NHEC-178 (Na^+/H^+ exchanger fusion protein), 10 μg of phosphatase, either PP1 or PP2B, and 0.1% BSA. NHEC-178 was replaced with an equal concentration of purified GST in control experiments. To ensure optimal phosphatase activity, the experiments including PP1 were supplemented with 1 mM $MnCl_2$ as a source of Mn^{2+} ions, while assays containing PP2B were supplemented with 5 mM $CaCl_2$, a source of Ca^{2+} ions. In addition, experiments investigating PP2B binding were performed in the presence of 5 and 10 μg of purified calmodulin (CaM).

Fifteen microliters of glutathione−Sepharose beads was added to each reaction mixture to immobilize the NHEC-178 protein. The reaction mixtures were incubated at 37 °C for 90 min with light shaking. Next, proteins were solubilized from the glutathione beads with 1× SDS−PAGE loading buffer and centrifuged at 14 000 rpm for 2 min at room temperature. The final supernatant was run on a 10% SDS gel, and transferred to a nitrocellulose membrane for Western blotting.

Immunoprecipitation of NHE1 from Cells Treated with Cross-Linking Reagents. For immunoprecipitation of NHE1, AP1-NHE1 cells were used. These are cells of a Chinese hamster ovary cell line that overexpresses human NHE1 with a hemagglutinin (HA) epitope tag (*32*). Cell lysates from AP1-NHE1 cells were prepared essentially as described previously (*12*) by incubating cells in 300 μL of RIPA lysis buffer lacking detergents 150 mM NaCl, 80 mM NaF, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and 1 μL/mL Protease inhibitor cocktail (*45*) on dry ice for 1 min before allowing them to thaw on ice. Once the cells thawed, they were scraped and transferred to an Eppendorff tube. The cells were centrifuged at 14 000 rpm for 15 min at 4 °C. The supernatant was discarded, while the pellet, containing the Na^+/H^+ exchanger, was resuspended in 1 mL of RIPA lysis buffer containing detergent [1.0% (v/v) NP-40, 0.5% (w/v) deoxycholate, and 0.1% (w/v) SDS] and sonicated for 20 s. Centrifugation was repeated at 14 000 rpm for 1 h at 4 °C (*32*). Finally, 1 mL of the supernatant was stored in liquid nitrogen for later use in immunoprecipitation studies.

Cross-linking was performed prior to immunoprecipitation of NHE1, to determine whether PP1 or PP2B could physically interact with NHE1. The cells were treated with DSP [dithiobis(succinimidylopropionate)] according to the manufacturer's instructions (Pierce). DSP was dissolved in dimethyl sulfoxide (DMSO) immediately prior to use. Cells were washed twice with PBS and incubated with 2 mM DSP for 30 min at room temperature. The reaction was quenched with 20 mM Tris-HCl (pH 7.5) for 15 min. Cell lysates of AP1-NHE1 cells treated with cross-linking reagents were made as described above.

For immunoprecipitation, cell lysates were thawed on ice and 100 μL of lysate was retained for a positive control. Immunoprecipitation was with 30 μL of rabbit polyclonal anti-NHE1 antibody and was essentially as described previously (*12*). After immunoprecipitation, protein A−Sepharose beads containing immunoprecipitated proteins were suspended in 40 μL of 4× SDS−PAGE loading buffer and initially incubated at 37 °C for 15 min followed by a second 15 min incubation at 60 °C. A 10 μL aliquot was run on a 10% SDS−PAGE gel, and subsequently transferred to

nitrocellulose for Western blotting with either an anti-mouse monoclonal primary antibody against NHE1 (Chemicon) or a polyclonal anti-PP1α, a monoclonal anti-PP1, or a polyclonal anti-calcineurin antibody (Santa Cruz Biotechnology).

Assay of Phosphatase Activity. The level of phosphatase (PP1 or PP2A) activity was examined in extracts of cell lines expressing I2 protein or PP1. Glycogen phosphorylase *a* was used as a substrate and was prepared and used as described previously (*46*) with the following modifications. Fifty milligrams of glycogen phosphorylase *b* (Sigma) was resuspended in 800 μL of H_2O and dialyzed overnight into 50 mM Tris (pH 7.0), 1 mM EDTA, 25 mM NaF, and 0.1% 2-mercaptoethanol. One kilounit of phosphorylase kinase (Sigma) was resuspended in 800 μL of H_2O and dialyzed overnight in the same buffer as the phosphorylase *b*. Phosphorylase *b* was then concentrated to a volume of 200 μL using Centricon 30 concentrators (Amicon). The phosphorylase *b* and phosphorylase kinase were then incubated for 60 min at 30 °C in 18.5 mM magnesium acetate, 12.4 mM sodium glycerol 1-phosphate, 0.23 mM calcium chloride, 0.33 mM cold ATP, and 1 mCi of [γ-^{32} P]ATP (Pharmacia). The total reaction volume was approximately 1.5 mL. An equal volume of cold saturated ammonium sulfate was then added, and the reaction mixture was placed on ice for 15 min. The suspension was then centrifuged at 16000*g* for 15 min, and the supernatant was discarded. The precipitate was resuspended in 600 μL of buffer containing 50 mM Tris (pH 7.0), 1 mM EDTA, and 0.1% 2-mercaptoethanol and dialyzed in this buffer at 4 °C to remove the remaining radioactive ATP. During this dialysis, the phosphorylase *a* crystallizes. The phosphorylase *a* is then resuspended in 4 mL of the dialysis buffer and aliquotted into 100 μL aliquots. Prior to use, one aliquot is diluted to 400 μL with 50 mM Tris (pH 7), 0.1 mM EDTA, 0.5 mM $MnCl_2$, and 0.1% 2-mercaptoethanol containing 15 mM caffeine and stored on ice. Ten microliters of the resuspended phosphorylase *a* was used in each reaction in a reaction buffer containing 50 mM Tris (pH 7), 0.1 mM EDTA, 0.5 mM $MnCl_2$, and 0.1% 2-mercaptoethanol.

For phosphorylase *a* assays, cell lysates were prepared as follows. Cells were grown in 60 mm Petri dishes to 90% confluency. Cells were washed several times with 1× TBS containing the following protease inhibitors: aprotinin (0.5 μg/μL), leupeptin (1.0 μg/μL), pepstatin (1.0 μg/μL), and phenylmethanesulfonyl fluoride (1 mM). Cells were replenished with 100 μL of harvesting buffer (50 mM Tris-HCl and 1 mM EDTA) and detached from the dish surface with a disposable cell scraper. The cells were centrifuged at 14 000 rpm for 5 min at 4 °C in microfuge tubes, and the supernatant was transferred to a separate vial. Fresh harvesting buffer (50 μL) was added to the pellet that contained the protein, and the pellet was dispersed to make a homogeneous solution. The sample was frozen in liquid nitrogen for use in phosphorylase *a* assays.

RESULTS

In Vitro Dephosphorylation of NHE1. A GST−Na^+/H^+ exchanger fusion protein consisting of the C-terminal 178 amino acids of the NHE1 isoform (NHEC-178) (*34*) was employed in in vitro dephosphorylation experiments. The protein was phosphorylated using heart cell extracts as previously described (*12*). The dephosphorylation of NHE1 was investigated using purified protein phosphatases PP1, PP2A, and PP2B. The phosphorylated NHE1 protein was incubated with each purified phosphatase (PP1, PP2B, or PP2A) under the appropriate conditions required to maintain their activity. Figure 1a (PP1) shows a representative autoradiogram of the incubation of phosphorylated NHEC-178 with PP1 and demonstrates that PP1 abolished the radioactive phosphate signal. In control reactions, the ^{32}P signal remained (Figure 1a, Ctrl). A quantitative analysis of these experiments using the ^{32}P:protein ratio confirms that PP1 removed almost all of the ^{32}P from the protein (Figure 1c).

Dephosphorylation of NHE1 by PP2A was carried out in the presence or absence of Mn^{2+} (Figure 1d−f). A quantitative analysis of the ^{32}P:protein ratios revealed significant dephosphorylation of NHE1 by PP2A both in the presence and in the absence of Mn^{2+} (Figure 1f); however, in the absence of Mn^{2+} (Figure 2a, $-Mn^{2+}$), the magnitude of the signal was decreased from that observed in the presence of Mn^{2+} (Figure 1d, $+Mn^{2+}$). Dephosphorylation of NHE1 by PP1 is significantly more complete than that by PP2A, as demonstrated in Figure 1d (PP1) and quantified in Figure 1f.

Incubation of phosphorylated NHEC-178 with PP2B in the presence of Ca^{2+} and CaM did not result in dephosphorylation of the protein. Figure 1g shows that the ^{32}P signal was comparable to that observed in the control reaction (Figure 1f, Ctrl), which lacked PP2B. Identical results were observed in the absence of CaM (data not shown). A quantitative analysis of the ^{32}P:protein ratios confirmed that PP2B did not dephosphorylate NHE1 significantly (Figure 1i).

Effects of Okadaic Acid on NHE1 Activity in Isolated Cardiomyocytes. We examined which protein phosphatases could be important in regulating NHE1 activity in isolated cardiomyocytes by treating the cells with the phosphatase inhibitor okadaic acid (*30*). Figure 2a illustrates an example of pH measurement in AP1 cells. It shows the ammonium chloride induced acidification of the cells, followed by recovery from an acid load and calibration of the intracellular pH. Okadaic acid inhibits PP2A at sub-nanomolar concentrations, while it is a less potent inhibitor of PP1 and does not inhibit PP2B (*21*). To test the effects of okadaic acid, isolated cardiomyocyte cells were treated with an ammonium chloride-induced acid load as described in Experimental Procedures. The rate of recovery from acid load was measured in the presence of 1 μM okadaic acid. Figure 2b shows one example of the effects on recovery of intracellular pH, while Figure 2c shows a summary of the results. Okadaic acid significantly stimulated the initial rate of recovery from an acid load. Low concentrations of okadaic acid (1 nM) did not stimulate Na^+/H^+ exchanger activity in this system (not shown), suggesting that PP2A was not a critical phosphatase in this system (*20*).

In Vitro Interactions of NHE1 with Protein Phosphatases. Pull-down assays were used to examine the interactions of the C-terminus of NHE1 (NHEC-178) with PP1 and PP2B. NHEC-178 or a control (GST) was immobilized on glutathione−Sepharose beads and incubated with PP1 and Mn^{2+} or PP2B and Ca^{2+} and CaM, at 37 °C. Pelleting the beads terminated the reactions, and any attached complexes were analyzed by Western blotting with a polyclonal antibody

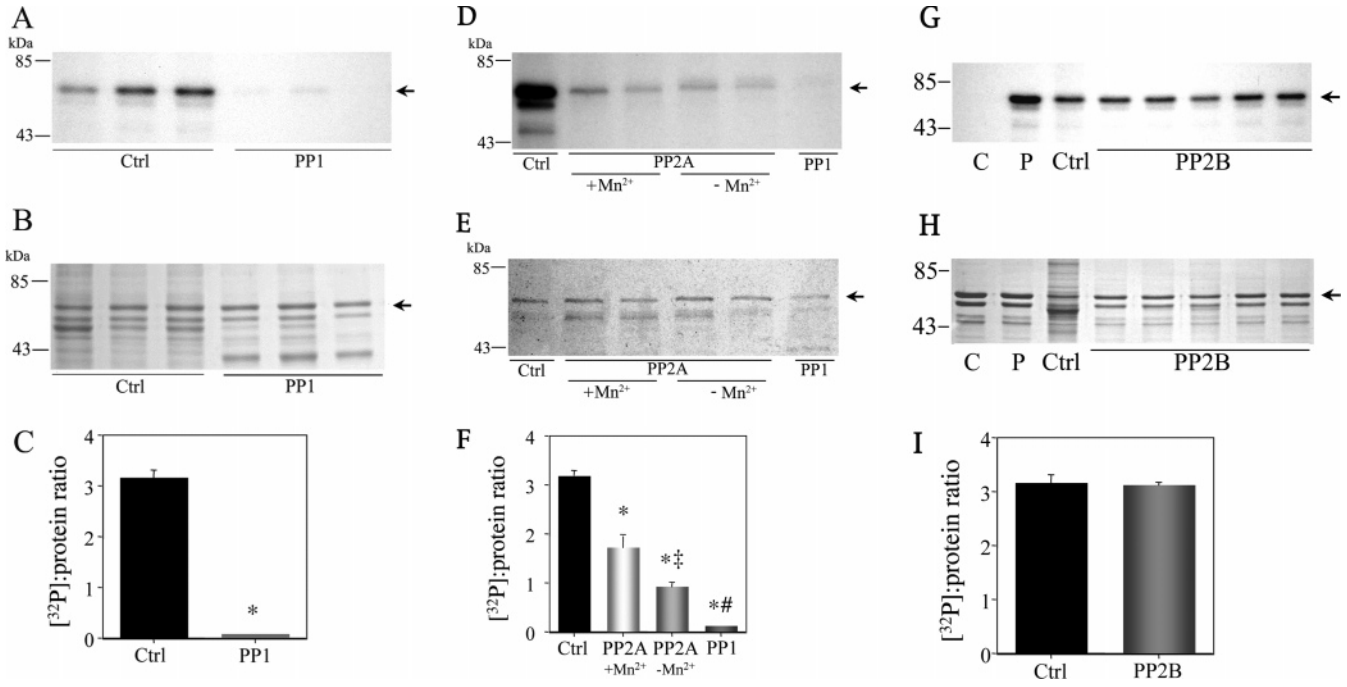

FIGURE 1: Dephosphorylation of the Na^+/H^+ exchanger by protein phosphatases. (A−C) PP1 dephosphorylation of the Na^+/H^+ exchanger. (A) Representative autoradiogram of dephosphorylation of the C-terminal Na^+/H^+ exchanger fusion protein (NHEC-178, arrow). In PP1 lanes, experimental samples were treated with 10 μg of PP1 and 5 mM $MnCl_2$. In Ctrl lanes, control samples were treated the same as experimental samples except in the absence of PP1. (B) Coomassie blue-stained SDS−PAGE gel illustrating the amount of NHEC-178 (arrow) present in each dephosphorylation reaction mixture. (C) Quantitative analysis of the ^{32}P:protein ratio in the presence (PP1) or absence (Ctrl) of PP1. The asterisk indicates a result significantly different from the control at $P < 0.01$. Results are the mean ± the standard error of six experiments. Where not shown, the standard error is too small to be displayed. (D−F) Dephosphorylation of NHE1 by PP2A in the presence and absence of Mn^{2+}. (D) Representative autoradiogram of dephosphorylation of the C-terminal cytoplasmic Na^+/H^+ exchanger fusion protein (NHEC-178, arrow) by 10 μg of PP2A in the presence ($+Mn^{2+}$) or absence ($-Mn^{2+}$) of 5 mM Mn^{2+}. Control (Ctrl) dephosphorylation reactions lacked PP2A. Dephosphorylation in the presence of PP1 was carried out as described for panel A (PP1). (E) Coomassie blue-stained SDS−PAGE gel illustrating the amount of NHEC-178 (arrow) present in dephosphorylation reaction mixtures. (F) Quantitative analysis of the ^{32}P:protein ratio in the presence of PP2A (with or without 5 mM Mn^{2+}) or PP1 or in the absence of phosphatase (Ctrl). Asterisks indicate significant dephosphorylation ($P < 0.05$) in comparison to controls. The double dagger indicates a value significantly different from that of PP2A with Mn^{2+} ($P < 0.05$). A number sign indicates a value significantly different from that of PP2A without Mn^{2+} ($P < 0.05$). Results are the mean ± the standard error of at least four experiments. (G−I) NHE1 dephosphorylation by PP2B. (G) Representative autoradiogram of dephosphorylation of the C-terminal cytoplasmic Na^+/H^+ exchanger fusion protein (NHEC-178, arrow) in the presence of 10 μg of PP2B, 10 mM $CaCl_2$, and 5 μg of calmodulin (PP2B). C indicates the NHEC-178 protein was not phosphorylated. The control (Ctrl) reaction lacked PP2B. P indicates phosphorylated NHEC-178 (not subjected to any dephosphorylation treatment). (H) Coomassie blue-stained SDS−PAGE gel illustrating the amount of NHEC-178 (arrow) present in each dephosphorylation reaction mixture. (I) Quantitative analysis of the ^{32}P:protein ratio in the presence (PP2B) and absence (Ctrl) of PP2B. Results are the mean ± the standard error of seven experiments.

against PP1 (Figure 3). As illustrated in Figure 3b, the extent of binding of PP1 to NHEC-178 was significantly greater than to GST alone. A similar experiment with PP2B failed to demonstrate any binding of intact full-length PP2B to NHEC-178 (not shown).

In Vivo Interactions of NHE1 with Protein Phosphatases. To examine the interactions between NHE1 and protein phosphatases in vivo, we used immunoprecipitated NHE1 from the AP1-NHE1 Chinese hamster ovary cell line. The Na^+/H^+ exchanger in AP1-NHE1 cells contains an HA epitope tag (*32*). Cells were treated with DSP and immunoprecipitated with rabbit polyclonal, anti-NHE1 antibody as described in Experimental Procedures. The results are shown in Figure 4. Figure 4a shows that we successfully immunoprecipitated the Na^+/H^+ exchanger. Anti-NHE1 antibodies revealed two immunoreactive proteins, a cross-linked NHE1 protein (>203 kDa, top arrow) in addition to NHE1 (~115 kDa, bottom arrow). Earlier studies by others and ourselves have shown that NHE1 exists as a dimer (*47*, *48*). The upper dimeric form of the protein contained 67% of the total immunoreactivity. To detect possible binding of PP1 to NHE1, a monoclonal anti-PP1 antibody was used for immunoblot analysis. A positive control of purified PP1 produced in *E. coli* was 37 kDa in size and confirmed the specificity of the antibody that was used. A 32 kDa band was observed in samples of immunoprecipitated NHE1 from cells cross-linked with DSP (Figure 4b, DSP, arrow). The difference in size between the immunoprecipitated PP1 and the positive control was presumed to be due to processing of the protein in the eukaryotic cell or to proteolysis. This phosphatase has been shown (*49*) to be cleaved and activated by proteolysis. In the absence of DSP, we could not detect PP1 linked to the Na^+/H^+ exchanger (Figure 4b, −DSP). This indicated that the interactions between PP1 and NHE1 were not stable enough to be detected in the absence of the cross-linker.

To detect possible PP2B binding to NHE1, a polyclonal anti-PP2B antibody was utilized to examine the cross-linked immunoprecipitate (Figure 4c). PP2B binding was not detected in samples of immunoprecipitated NHE1. A positive control of PP2B on the immunoblot confirmed that the immunoblotting reaction was functioning. Overall, the results

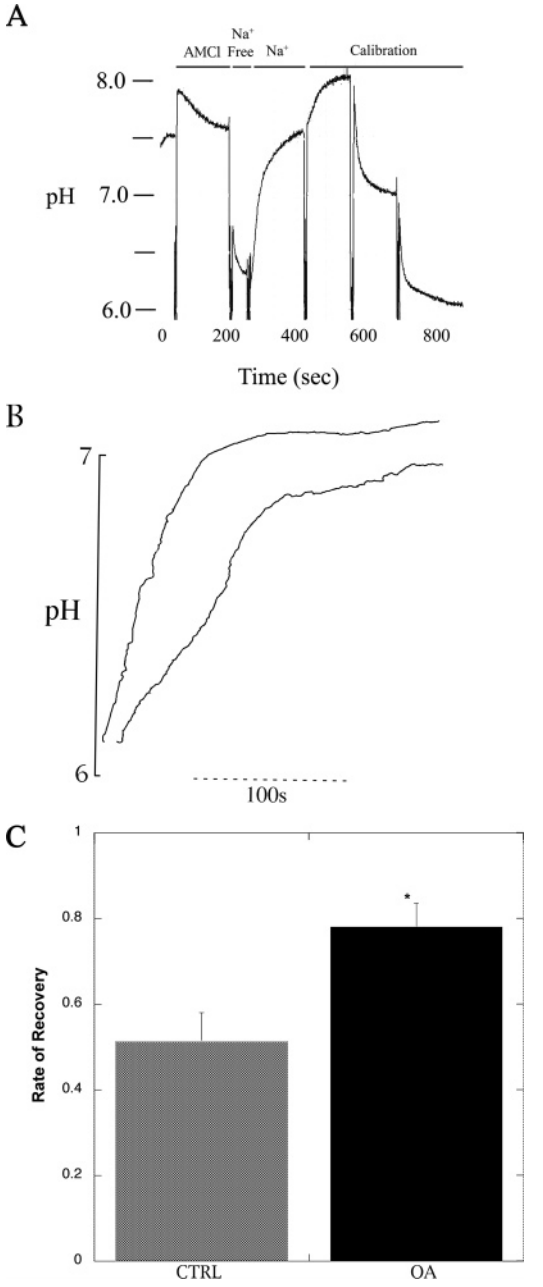

FIGURE 2: NHE1 activity in neonatal rat myocytes treated with the phosphatase inhibitor, okadaic acid. (A) Representative tracing of a typical assay measuring the rate of recovery from an acid load in mammalian cells. Acid load was induced using ammonium chloride (40 mM NH_4Cl) for 3 min followed by Na^+-free buffer. The rate of recovery was measured for 3 min in Na^+-containing buffer, followed by a three-point calibration in a nigericin-containing solution at pH 8, 7, and 6 (as described in Experimental Procedures). (B) Examples of recovery from ammonium chloride-induced acid load in isolated cardiomyocytes treated with 1 μM okadaic acid in Na^+-containing buffer. The intracellular pH was measured after ammonium chloride-induced acid load as described in the Experimental Procedures. Traces represent approximately 200 s of recovery. Only the recovery in Na^+-containing buffer after Na^+-free buffer is illustrated. The top trace depicts data for okadaic acid-treated cells. The bottom trace depicts data for control cells and was offset for easier viewing. (C) Summary of the effect of 1 μM okadaic acid on isolated cardiomyocytes treated as described for Figure 4B. The black column represents data for myocytes treated with okadaic acid immediately prior to recovery from acid load, and the gray column represents data for myocytes treated with vehicle alone. Results are the mean ± the standard error of at least five experiments. The asterisk indicates significant difference from control at $P < 0.05$.

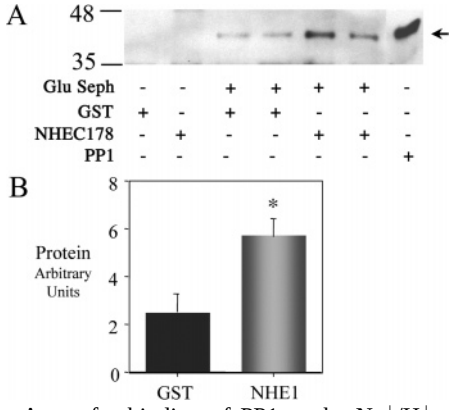

FIGURE 3: Assay for binding of PP1 to the Na^+/H^+ exchanger. GST or the Na^+/H^+ exchanger−GST fusion protein (NHEC-178) was incubated with PP1 protein. Any complexes that formed were recovered with glutathione−Sepharose beads, and samples were analyzed by Western blotting. (A) Western blot analysis of glutathione−Sepharose pellets probed with the anti-PP1 polyclonal antibody. Reactions were carried out in the presence or absence of glutathione−Sepharose (Glu Seph) beads as indicated. GST protein and NHEC-178 proteins were in a mock reaction in the absence of glutathione−Sepharose beads as negative controls (first two lanes). Glutathione−Sepharose pellets of GST incubated with PP1 were made as controls to obtain levels of nonspecific binding to the beads and GST. Glutathione−Sepharose pellets of NHEC-178 incubated with PP1 indicated binding to the tail of NHE1. A positive control was run on the gel containing purified PP1 alone (PP1+). (B) Quantitative analysis of GST pull-down experiments with PP1 and GST alone (GST), or PP1 with NHEC-178 (NHE1). The asterisk indicates the level of PP1 binding to NHEC-178 is significantly greater than that to GST at $P < 0.05$. Results are the mean ± the standard error of four experiments.

of immunoprecipitation experiments indicate that NHE1 associates with PP1 in vivo, while we were unable to detect an association of PP2B with the Na^+/H^+ exchanger.

Phosphorylase Phosphatase and PP1 Activity in I2 Cells. The level of PP1 activity was examined in the cell lines overexpressing I2 using a phosphorylase *a* assay as described previously (*50*). In two independently made cell lines expressing I2, the activity of PP1 was reduced by 67 and 79% in comparison with cells that were mock transfected with vector alone. For unknown reasons, attempts to assay phosphatase activity of PP1-expressing cell lines were not successful.

Effects of PP1 on Na^+/H^+ Exchanger Activity in Vivo. To examine the effects of PP1 on NHE1 activity in vivo, stable cell lines were made of CHO cells expressing either PP1 or I2, the specific inhibitor of PP1 activity. Control cells were CHO cells mock transfected with an empty vector. For unknown reasons, stable cell lines overexpressing PP2B were not successful despite a high transfection efficiency and screening of more than 30 colonies. Expression of PP1 and I2 in CHO cells was verified using a polyclonal anti-V5 antibody against extracts of colonies of each cell line. Figure 5a illustrates a Western blot typical of at least two cell lines expressing PP1 or I2. In both cases, an immunoreactive protein was apparent with the anti-V5 antibody. Cells transfected with the empty vector produced no immunoreactive protein. A series of experiments examined the effects of expression of PP1 and I2 on the level of expression of the Na^+/H^+ exchanger protein in transfected cell lines. Western blotting with anti-NHE1 antibody determined that expression of PP1 and I2 did not affect NHE1 expression levels (not shown).

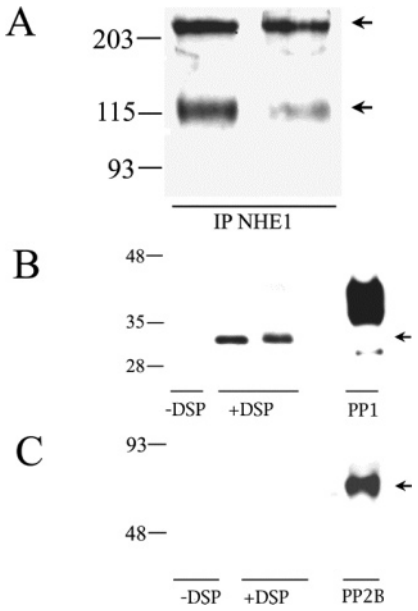

FIGURE 4: In vivo association of PP1 and the Na^+/H^+ exchanger. (A) Representative Western blot of the immunoprecipitated Na^+/H^+ exchanger from AP1-NHE1 cells treated with the cross-linker DSP. The Na^+/H^+ exchanger was immunoprecipitated with a rabbit polyclonal anti-NHE1 antibody, and Western blot analysis was carried out with a monoclonal anti-NHE1 antibody. The top arrow denotes cross-linked NHE1; the bottom arrow denotes the tagged Na^+/H^+ exchanger protein of 115 kDa. (B) Western blots probed with a monoclonal antibody against PP1. The Na^+/H^+ exchanger was immunoprecipitated from cell lysates of AP1-NHE1 cells either treated (+DSP) or not treated (−DSP) with DSP. A positive control of purified PP1 (PP1) was used to confirm the immunoreactivity of the antibody. (C) Western blot of immunoprecipitated NHE1 probed with a polyclonal anti-PP2B antibody. The Na^+/H^+ exchanger was immunoprecipitated from cell lysates of AP1-NHE1 cells either treated (+DSP) or not treated (−DSP) with DSP. A positive control of purified PP2B (PP2B) was used to confirm the immunoreactivity of the antibody.

We next examined the effects of PP1 and I2 expression on Na^+/H^+ exchanger activity. Figure 5b shows a summary of the effects on the rate of recovery from an acid load in cells expressing PP1. Proton efflux from cells expressing PP1 was approximately half that of control cells expressing the empty vector. Figure 5c summarizes the effect of thrombin on the rate of recovery from an acid load of control and PP1-expressing cells. Thrombin stimulated the rate of recovery in all the cell types, though that of the PP1-expressing cells was always lower than that of the controls. Figure 5d illustrates a summary of the effect of expression of I2 on H^+ efflux mediated by the Na^+/H^+ exchanger. Expression of I2 significantly stimulated the rate of recovery from an acid load. Figure 5e illustrates a summary of the effects of thrombin on the rate of recovery from an acid load. Thrombin stimulated the recovery of control cells; however, it did not stimulate the rate of recovery of cells expressing I2. The I2-expressing cells behaved as if they were already activated, and could not be further stimulated. Figure 5f shows examples of the initial rate of recovery from an acid load, after the sodium-containing solution was returned following ammonium chloride prepulse. The *X* axis is greatly expanded. The rate of recovery of I2-expressing cells is higher than that of control cells, and PP1-expressing cells have reduced rates of recovery in comparison to controls. Inclusion of the Na^+/H^+ exchanger inhibitor HMA completely blocks the recovery of pH in all the cell lines that were examined. An example of the effect of inhibition of Na^+/H^+ exchanger activity on the control cell line is illustrated.

DISCUSSION

Given the widespread distribution and important physiological and pathological functions of the Na^+/H^+ exchanger, studies investigating its regulation are of prime importance. While a fundamental understanding of the activation by agonists and protein kinases has begun, the requisite dephosphorylation that occurs after activation is not well-known. In this study, we examined three members of the PPP family (*28*) of protein phosphatases that were likely to play a role in dephosphorylation of the Na^+/H^+ exchanger. Three general approaches were used: (1) examination of the ability of phosphatases to dephosphorylate a phosphorylated protein in vitro, (2) examination of an association between phosphatases and the Na^+/H^+ exchanger in vivo, and (3) examination of the effects of protein phosphatases on the activity of the Na^+/H^+ exchanger in intact cells. Our initial experiments demonstrated that both PP1 and PP2A were able to dephosphorylate the Na^+/H^+ exchanger in vitro while PP2B did not significantly dephosphorylate the protein. Dephosphorylation of the Na^+/H^+ exchanger was more complete with PP1 than with PP2A, suggesting that it had a greater efficacy toward this protein. These results gave the first indications that PP1 could be a phosphatase that is important in regulation of the Na^+/H^+ exchanger.

Others have shown that okadaic acid can stimulate the Na^+/H^+ exchanger in distinct cell types, including elevation of activity in rat thymocytes (*10*) and blood platelets (*22*). Okadaic acid stimulates the Na^+/H^+ exchanger by phosphorylating a set of amino acids common to growth factor activation of the protein. The identical sites were phosphorylated to a greater degree by thrombin when treated in the presence of okadaic acid (*13*). However, okadaic acid does not stimulate the Na^+/H^+ exchanger of peripheral blood lymphocytes (*51*) and is relatively ineffective in stimulation of the trout isoform of the Na^+/H^+ exchanger (*25*). We tested the ability of okadaic acid to activate the Na^+/H^+ exchanger in isolated cardiomyocytes and demonstrated that it stimulated the rate of recovery from an acid load. Stimulation of the Na^+/H^+ exchanger by the concentrations of okadaic acid used in this study is purported to indicate that either PP1 or PP2A is the phosphatase involved in regulation (*13*). In other experiments, we found that lower concentrations of okadaic acid did not stimulate activity of the Na^+/H^+ exchanger. Therefore, PP2A, the phosphatase most sensitive to okadaic acid, was unlikely to be a key regulatory phosphatase in our system. For this reason, we did not further pursue studies on PP2A.

We further examined the role of PP1 in regulation of the Na^+/H^+ exchanger because of its greater efficacy in dephosphorylation of the protein and because some earlier studies had indirectly suggested that PP1 could be important in regulation of the Na^+/H^+ exchanger (*25*−*27*, *52*). To confirm that PP1 could regulate the Na^+/H^+ exchanger, we demonstrated that it can associate with the Na^+/H^+ exchanger both in vitro and in vivo. Protein phosphatases are known to bind to and dephosphorylate their target proteins. Examples include calcineurin binding to other membrane proteins such

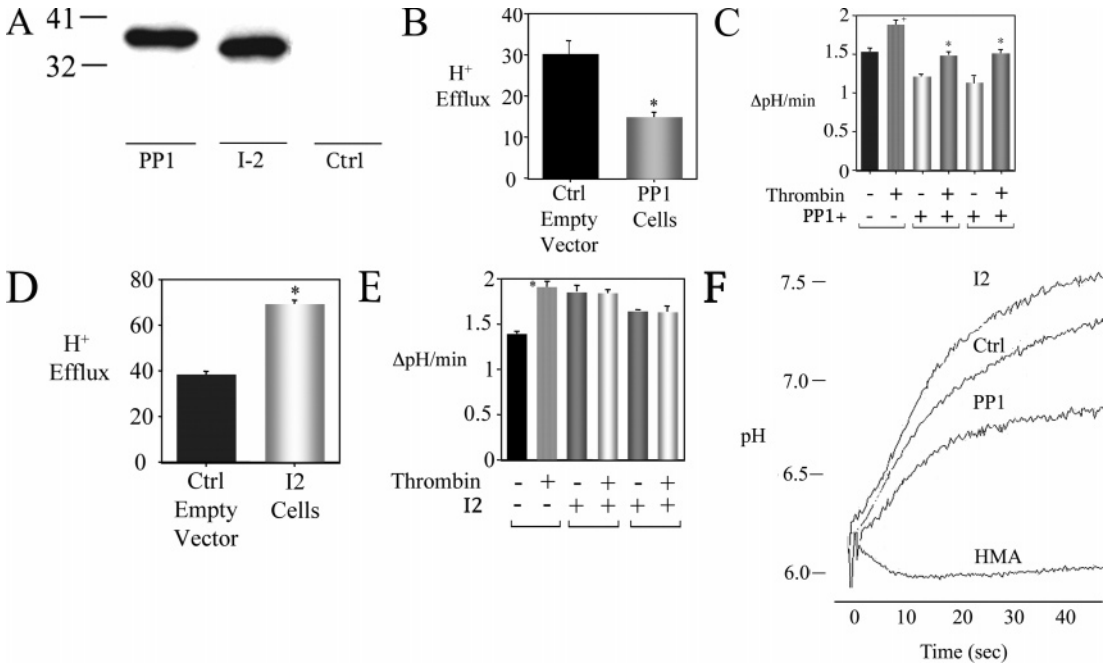

FIGURE 5: Expression of PP1 and I2 in CHO cells. (A) Western blot of stable cell lines expressing PP1 and I2. Cell extracts of representative colonies of stable cell lines expressing either PP1 (PP1) or I2 (I2) were probed with the anti-V5 antibody (epitope tag antibody). The Ctrl lane contained control cell lines transfected with an empty vector. (B) Effect of PP1 expression on the rate of recovery from an ammonium chloride-induced acid load. Control cells or cells expressing PP1 protein were grown to the same degree of confluence and were treated with an ammonium chloride-induced acid load. After measurement of the rate of recovery from an acid load, the proton efflux was calculated after using the buffering capacity of each cell type. The asterisk indicates a value significantly different from controls at $P < 0.05$; results are the mean ± the standard error of eight experiments. (C) The rate of recovery from an ammonium chloride-induced acid load was measured in the presence or absence of 2 units of thrombin/mL. Results are shown for two cell lines expressing PP1 (PP1+) and for control cells not expressing PP1 (PP1−). Thrombin was added where indicated (Thrombin+). All results are the mean ± the standard error of at least eight experiments. Asterisks and plus signs indicate values significantly different from the same cell type in the absence of thrombin at $P < 0.05$ and $P < 0.01$, respectively. (D) Effect of I2 expression on the rate of recovery from an ammonium chloride-induced acid load. Control cells and cell lines expressing the I2 protein were grown to the same degree of confluence and were treated with an ammonium chloride-induced acid load. After measurement of the rate of recovery from an acid load, the proton efflux was calculated using the buffering capacity of each type of cell. The asterisk indicates a value significantly different from controls at $P < 0.01$. Results are the mean ± the standard error of eight experiments. (E) Effect of I2 expression on thrombin-stimulated Na^+/H^+ exchanger activity. The rate of recovery from an ammonium chloride-induced acid load was measured in the presence or absence of 2 units of thrombin/mL. Results are shown for a control cell line (−I2) and for two cell lines expressing I2 (+I2). Cells were treated with thrombin as indicated (+). All results are the mean ± the standard error of at least nine experiments. The asterisk indicates a value significantly different from the same cell type in the absence of thrombin at $P < 0.01$. (F) Representative tracings of the recovery from an ammonium chloride-induced acid load in cells stably expressing I2 (I2) or PP1 (PP1) and controls (with the *X* axis expanded). Specific NHE1 inhibitor hexamethylene amiloride (HMA) was used to inhibit Na^+/H^+ exchanger activity.

as the IP_3 receptor (*53*) and the ryanodine receptor (*54*, *55*) and PP2A association with Bcl2 (*56*). PP1 has been shown to bind to other proteins such as p130 (*57*) and the Na^+/Ca^{2+} exchanger; however, the interactions may be mediated by PP1-binding proteins such as spinophilin (*58*). We showed that, in addition to binding to the Na^+/H^+ exchanger in vitro, PP1 was associated with the intact full-length Na^+/H^+ exchanger in vivo. In this instance, we have not yet determined whether an intermediate protein targeting PP1 to NHE1 is involved. The C-terminal tail of the NHE1 Na^+/H^+ exchanger comprises 315 residues, and we presume that PP1 must bind to this region of the exchanger. PP1 is known to associate with a large number of different polypeptides that function as substrates, inhibitors, chaperones, and anchoring or scaffolding proteins (*59*, *60*). The most well-characterized PP1 interactor binding site is the hydrophobic "RVXF" binding groove, which is distinct from the active site (*61*). Most PP1-interacting proteins contain an RVXF motif sequence that binds to this hydrophobic channel (*59*, *60*). This sequence is lacking in the NHE1 exchanger; however, other modes of interaction have been identified for PP1-binding proteins that do not require this specific motif, for example, sds22 which binds to PP1 at a different site (*62*) or inhibitor 2 which comprises multiple sites of binding (*63*, *64*). The exact motif to which PP1 binds in the Na^+/H^+ exchanger has yet to be determined.

Since we had shown that PP1 both can dephosphorylate the Na^+/H^+ exchanger in vitro and is associated with the Na^+/H^+ exchanger in vitro and in vivo, we examined the effects of overexpression of this phosphatase and of the specific PP1 inhibitor, I2. Expression of PP1 inhibited the activity of the Na^+/H^+ exchanger, while expression of I2 stimulated the Na^+/H^+ exchanger (Table 2). An interesting effect of the expression of I2 was that the cells behaved as if they were already activated, and could not be further stimulated by thrombin (Figure 5e and Table 2). This result is consistent with a regulatory role of PP1 in the removal of phosphates that would normally be present through thrombin-induced phosphorylation. Because I2 is a very specific inhibitor of PP1 (*63*, *64*), the fact that I2 expression stimulates Na^+/H^+ exchanger activity strongly supports the conclusion that PP1 is a critical phosphatase regulating the Na^+/H^+ exchanger. I2 inhibition of phosphatase activity and stimulation of the Na^+/H^+ exchanger were similar to the

Table 2: Summary of Effects of Pharmacological and Biochemical Interventions on Phosphatase Activity and Na^+/H^+ Exchanger Activity[a]

intervention	Na^+/H^+ exchanger[b]	Na^+/H^+ exchanger[c] (thrombin stim)	phosphatase activity
okadaic acid treatment	+	N/A	−
PP1 expression	−	+	+
I2 expression	+	−	−

[a] Isolated cardiomyocyte cells were treated with okadaic acid as described in the legend of Figure 4, and the rate of recovery from an ammonium chloride-induced acid load was measured. In other experiments, Na^+/H^+ exchanger activity of stable cell lines of CHO cells expressing either PP1 or I2 was also characterized. [b] Na^+/H^+ exchanger activity induced by ammonium chloride-induced acidosis. [c] Ability of thrombin to stimulate the Na^+/H^+ exchanger in stable cell lines expressing PP1 or I2.

effect of the phosphatase inhibitor okadaic acid on Na^+/H^+ exchanger activity (Table 2).

Whether PP1 is responsible for removal of all or a selective group of the phosphates on the C-terminal tail of the Na^+/H^+ exchanger is not known at this time. In our in vitro studies, PP1 removed virtually all the phosphate from the phosphorylated protein, while PP2A did not completely dephosphorylate the protein. Since low concentrations of okadaic acid did not stimulate activity of the Na^+/H^+ exchanger, we did not investigate this phosphatase further. It may be that it is important in other cell types (*13*). Future studies may investigate the role of this phosphatase. It should be noted that several different amino acids of the cytosolic domain of the Na^+/H^+ exchanger have been reported to be phosphorylated by protein kinases $p90^{rsk}$ and p38, including Ser 703, Thr 717, Ser 722, Ser 725, and Ser 728 (*17*, *18*). In addition, Ser 693, Ser 766, Ser 770, Thr 779, and Ser 785 are phosphorylated by Erk1 (*65*). It may be that one type of phosphatase acts on some of the sites and that another acts on other sites. Possibly this is tissue specific. Future experiments will address this possibility.

In summary, this is the first report demonstrating the effects of a protein phosphatase on the Na^+/H^+ exchanger in vivo. We show that PP1 binds to, dephosphorylates, and regulates the activity of the Na^+/H^+ exchanger. The involvement of other protein phosphatases cannot be excluded at this time; however, a role for PP1 has been established. Future experiments will examine how this phosphatase regulates the Na^+/H^+ exchanger during different physiological events and in various tissues. In addition, the role of protein phosphatases in regulating the Na^+/H^+ exchanger during hypertrophy and during ischemia and reperfusion may be examined.

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