Phosphorylation and Regulation of the Na^+/H^+ Exchanger through Mitogen-Activated Protein Kinase†

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Received April 7, 1997; Revised Manuscript Received May 22, 1997⊗

ABSTRACT: We examined mitogen-activated protein kinase-mediated phosphorylation and activation of the Na^+/H^+ exchanger isoform type 1. A rabbit skeletal muscle extract was fractionated by FPLC chromatography. Four main fractions had the ability to phosphorylate the carboxyl-terminal region of NHE1. Western blot analysis and immunoprecipitation showed that three of these were associated with MAP kinase-dependent phosphorylation. Phosphorylation studies using purified MAP kinase showed that the region involved was the carboxyl-terminal 178 amino acids of the protein and that the stoichiometry was 1 phosphate/mol of protein. In-gel kinase assays showed that cytosolic extracts from smooth muscle cells also phosphorylate the carboxyl-terminal of NHE1 and that the MAP kinase-dependent phosphorylation could be activated by PDGF and AngII. Mutant cell lines with an inducible dominant negative MAP kinase showed decreased serum activation of Na^+/H^+ exchange but normal hypertonic activation of the protein. The results show that MAP kinase is intimately involved in regulation of the Na^+/H^+ exchanger, possibly through phosphorylation of one amino acid of the carboxyl-terminal cytosolic domain.

The Na^+/H^+ exchanger is an almost universally distributed protein in mammalian cells. It has been implicated in a number of cellular functions, including pH regulation, cell volume regulation, and cell proliferation. There are several tissue-specific isoforms of the exchanger (NHE1-NHE5)[1] , which vary in molecular weight and in their sensitivity to the inhibitor amiloride (*1*). The amiloride-sensitive isoform (NHE1) is inhibited by nanomolar concentrations of amiloride analogs such as ethylisopropylamiloride. It extrudes one proton in exchange for one sodium ion when decreases in intracellular pH occur (*2*). The amino acid sequence of NHE1 has been deduced from the nucleotide sequence of a cDNA clone (*3*). The protein is glycosylated (*4*) and has an apparent molecular mass of about 100 kDa. The predicted sequence of the protein is highly conserved. The NHE1 isoform is often referred to as the ubiquitous or "housekeeping" form (NHE1) and exists in the plasma membrane of most cells. NHE1 is found in the basolateral membrane of most epithelial cells.

A great deal of effort has been made to determine the mechanisms of regulation of this protein. Early studies with intact cells showed that growth factors induce phosphorylation and activation of the Na^+/H^+ exchanger (*5*). The large carboxyl-terminal domain of the protein is a likely site of such regulation, being cytoplasmic and therefore accessible to the action of intracellular kinases (*3*, *5*). Wakabayashi *et al.* (*6*) have shown that growth factor activation is associated with phosphorylation of the C-terminal 179 amino acids of the NHE1 isoform. In the NHE3 isoform, different domains are believed to be either inhibitory or stimulatory of the activity of that isoform of the Na^+/H^+ exchanger (*7*). We have expressed part of the cytoplasmic domain of the protein in *Escherichia coli* and have shown that it is readily phosphorylated *in vitro* by Ca^{2+}/calmodulin-dependent protein kinase II. This region of the protein was not a substrate for purified protein kinase C or for the catalytic subunit of cAMP-dependent protein kinase (*20*). However, recently, Azarani *et al.* (*8*) showed that protein kinase A is involved in modulating Na^+/H^+ exchanger activity in Osteoblastic cells (UMR-106). It has also been suggested that a protein cofactor may be important in mediating some aspects of Na^+/H^+ exchanger regulation; however, the exact mechanisms and proteins involved have not been clarified (*9*, *10*).

In this study, we examined the putative role of mitogen-activated protein kinases (MAP Kinases) in regulation of the NHE1 isoform of the Na^+/H^+ exchanger. MAP kinases have been shown to be important in fibroblast proliferation stimulated by growth factors (*11*). In addition growth factors

† This work was supported by a grant from the Heart and Stroke Foundation of Canada.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

[1] Abbreviations: ANGII, angiotension II; GST, glutathione-S-transferase; HMA, 5-(*N*,*N*-hexamethylene)Amiloride; IGKA, in-gel kinase assay; IPTG, isopropyl-β-D-thiogalactopyranoside; MAP, mitogen-activated protein; NHE1, Na^+/H^+ exchanger isoform 1; $p44^{mpk}$, seastar meiosis-activated protein kinase; PBS, phosphate buffered saline; PCR178, PCR272, PCR343, GST fusion protein with carboxyl-terminal 178, 272, and 343 amino acids from the rabbit Na^+/H^+ exchanger; PDGF, platelet-derived growth factor; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WKY, Wistar-Kyoto.

S0006-2960(97)00802-7 CCC: $14.00

have been shown to activate the Na^+/H^+ exchanger in a number of studies (reviewed in ref 12). Here, we show that MAP kinase can directly phosphorylate the cytoplasmic region of the Na^+/H^+ exchanger. In addition, we showed that depletion of MAP kinase activity results in reduction of the ability of serum and growth factors to stimulate the protein. The results implicate MAP kinases in activation of the NHE1 isoform of the Na^+/H^+ exchanger.

MATERIALS AND METHODS

Materials. Molecular weight markers, cyanogen bromide-activated Sepharose 4B, plasmid pGEX-3X, and the Glutathione Sepharose 4B affinity column were from Pharmacia LKB. Horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Bio/Can (Mississauga, Ontario). Sodium dodecyl sulfate−polyacrylamide (SDS−PAGE) gels were prepared with reagents from Bio-Rad or Boehringer Mannheim, Inc. Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH). Synthetic oligonucleotides were synthesized with an Applied Biosystems, Model 392 DNA/RNA synthesizer, and DNA sequencing was done with an Applied Biosystems, Model 373A DNA sequencer in the Department of Biochemistry, DNA sequencing lab, University of Alberta. Anti MAP kinase R2 (Erk1-CT) and purified MAP kinase from sea star oocytes were from Kinetek Pharmaceuticals, Inc. (Vancouver, British Columbia). Anti MAP kinase R2 antibody is specific for the 43, 42, and 44 kDa kinases encoded by erk1, erk2, and mpk genes, respectively. All other chemicals were obtained commercially, and were of the highest available grade.

Polyacrylamide Gels and Immunostaining. SDS−PAGE was on 9% polyacrylamide gels (or on 7% gels as indicated) as described earlier (*13*). Gels were stained with Coomassie blue. For immunostaining, proteins were transferred electrophoretically onto nitrocellulose membranes. Immunostaining of nitrocellulose membranes was carried out in the presence of 1% skim milk powder using affinity-purified anti NHE1 antibodies essentially as described earlier (*14*), or by using the Amersham Enhanced Chemiluminescence Western Blotting and Detection System as described by the manufacturer.

Preparation of Glutathione-S-Transferase-Na^+/H^+ Exchanger Fusion Proteins. For production of carboxyl-terminal Na^+/H^+ exchanger fusion proteins, we expressed a series of proteins of the rabbit Na^+/H^+ exchanger cDNA clone. cDNA of the carboxyl-terminal 178 amino acids of the rabbit cardiac Na^+/H^+ exchanger was prepared by polymerase chain reaction (PCR), using the primers 5′ GCG GAT CCT GCA GAA GAC CCG GCA GCG GCT 3′ and 5′ AAG AAT TCT ACT GCCC (C/T)TT GGG GA(A/T) GAA, designed from bp 510−533 and 1027−1047 of the rabbit cardiac Na^+/H^+ exchanger clone described earlier (*15*). (The redundancies present in some primers are for use with the human Na^+/H^+ exchanger cDNA.) The fragment generated was cloned into the *Bam*H1−*Eco*R1 sites of expression plasmid pGEX-3X to generate PCR178. Two other fusion proteins were prepared using a similar procedure except that the first primer was replaced with the primers 5′ ACG GAT CCA TTG GAA (A/G)GA CAA GCT CAA CCG GTTT A 3′ and 5′ AAG TTA ACT TCC CCA TGT G(C/T)G ACC TGT TCC TCA C 3′ to generate fusion proteins of 272 (PCR272) and 343 (PCR343) amino acids respectively. PCR products were sequenced to confirm the correct amplification of the product. The glutathione-S-transferase-Na^+/H^+ exchanger fusion proteins were expressed in *E. coli* DH5-α, after induction with 0.2 mM IPTG. Cells were lysed with a French-press and the supernatant fraction (10000*g* × 10 min) containing fusion protein was analyzed by SDS−PAGE as described above.

Purification of Carboxyl-Terminal Na^+/H^+ Exchanger Fusion Proteins. Purification of fusion proteins was via glutathione Sepharose 4B affinity chromatography. All steps were performed at 4 °C in the presence of a cocktail of protease inhibitors (*16*). Samples were dialyzed against phosphate buffered saline (PBS) containing 1% Triton X-100 and protease inhibitors and were applied to the column at least twice. The column was washed with several bed volumes of PBS, and the samples were eluted with 5 mM glutathione. The column was prewashed with several volumes of buffer containing 3 mM MgATP prior to elution with glutathione to remove copurifying DNA K (*13*). On some occasions samples from the cell lysates were partially purified using preparative SDS−PAGE prior to purification with GST affinity chromatography. On these occasions, 1−5 mg of sample was size fractionated with a Bio-Rad Model 491 Prep Cell. The composition of the electrophoresis buffer and gel were as described above for SDS−PAGE. The appropriate fractions were identified by immunoblotting and pooled, concentrated, and the buffer was exchanged for PBS with 1% Triton X-100 and protease inhibitors. After renaturation in PBS, these fractions were then purified via glutathione Sepharose 4B affinity chromatography as described above.

Preparation and Fractionation of Soluble Extracts from Rabbit Skeletal Muscle. Muscle extracts were prepared from the red muscles of the back and hind limbs of New Zealand white rabbits. After sacrifice, the muscle tissue was immediately homogenized at a medium setting with a Polytron homogenizer for 30 s in 2.5 vol. of buffer containing 10 mM sodium phosphate pH 7.5, 60 mM β-glycerophosphate, 1 mM DTT, 15 mM EGTA, 4 mM EDTA, 1 uM okadaic acid, and a protease inhibitor cocktail (*16*). The homogenate was then centrifuged at 6000*g* for 60 min at 4 °C. The supernatant was filtered through glass wool and centrifuged at 100000*g* for 1 h at 4 °C. The supernatants (soluble muscle extracts) were stored in 50% glycerol at 4 °C. The soluble muscle extracts were fractionated on a Mono Q 10 column using FPLC chromatography. The extracts were applied to the column, and bound proteins were eluted with a 0−0.8 M linear NaCl gradient in 10 mM MOPS pH 7.2, 2 mM EDTA, and 1 mM DTT. Fractions (0.5 mL) were collected and analyzed by Western blot analysis and for their ability to phosphorylate expressed regions of the Na^+/H^+ exchanger.

Phosphorylation of Fusion Proteins. The standard reaction conditions for phosphorylation of muscle extract fractions contained 0.5 μg of PCR 343, 20 μL of muscle extract, 3 mM $MgCl_2$, and 25 μM ATP (containing approximately 1000 cpm/pmol of [γ-^{32}P]ATP) in a final volume of 30 μL. Samples were incubated at 37 °C for 60 min, and the reaction was terminated by the addition of loading buffer.

For studies with purified MAP kinase, the media for phosphorylation consisted of 20 mM MOPS buffer, pH 7.2, 0.8 mM EGTA, 3.2 mM DTT, 13.44 mM $MgCl_2$, 0.08 mM NaF, 75−250 ng of fusion protein, and 30 ng of purified sea star oocyte MAP kinase were added to each 25 μL

reaction. Reactions were initiated by the addition of ATP [0.08 mM [γ-^{32}P]ATP (approx. 110 cpm/pmol)] and were incubated at 30 °C. Samples were withdrawn at times indicated and added to SDS−PAGE gel sample buffer. They were examined by SDS−PAGE and autoradiography. Stoichiometry of phosphorylation was determined by using measured amounts of sample separated by SDS−PAGE. The appropriate bands were identified by autoradiography and excised. ^{32}P incorporation was measured by liquid scintillation counting and comparison to a standard curve generated with the reaction solution containing [γ-^{32}P]ATP. In some cases, the incorporated radioactivity was quantified using a model BAS1000 phosphoimager (Fuji Photo Film Co., Ltd.) and standard curves were made with dilutions of [γ-^{32}P]ATP.

For one set of experiments, in-gel kinase assays (IGKA) were used to examine *in vitro* phosphorylation of the Na^+/H^+ exchanger C-terminal region. The assays were performed essentially as described earlier (*17*), using the carboxyl-terminal region (PCR343) of the Na^+/H^+ exchanger incorporated into the gel (600 μg/5 mL). Primary cultures of vascular smooth muscle cells were obtained from 10−12 week Wistar−Kyoto (WKY) rats essentially as described earlier (*17*). Animal procedures conformed to the Guide for Care and Use of Laboratory Animals issued by the US Institute for Laboratory Resources. For all experiments cells, were plated in 60 mm dishes and growth arrested at 70−80% confluence for 48 h by reducing serum to 0.4%. Cells were treated with angiotension II (100 nM) or platelet-derived growth factor (PDGF, 10 ng/mL) as indicated prior to harvest.

Immunoprecipitation. For immunoprecipitation, purified anti-MAP kinase (R2) antibody was used. Anti-MAP kinase antibody (Kinetek Pharmaceuticals Inc.) was prebound to 20 μL of prewashed Protein A (Protein A, 10% v/v nonviable *Streptomyces aureus*, Sigma) extract in IPB buffer (20 mM Tris pH 7.8, 1 mM EDTA 650 mM NaCl, 0.5% NP40, 0.1% azide and 0.1% SDS) overnight at 4 °C. The antibody protein A complex was washed three times with IPB buffer to remove unbound antibody before addition to the protein samples. The complex was incubated with each fraction for 2 h at 4 °C. The complex was harvested by centrifugation at 350*g* for 1 min. The supernatant designated MAP kinase depleted fraction was used to phosphorylate PCR343. The pellet was washed five times with IPB buffer and resuspended in loading buffer for SDS−PAGE. IPB buffer was supplemented with a cocktail of protease inhibitors as described above.

Construction of Inducible, MAP Kinase Dominant Negative Cells. To examine the role of MAP kinase in activity of the Na^+/H^+ exchanger *in vivo* we constructed a MAP kinase inducible dominant negative mutant. We constructed an expression vector using the catalytically compromised version of p44^{erk1} in which Lys-71 was converted to Ala by site-directed mutagenesis (*18*). The defective mutant DNA was removed from pGEX-2T (*18*) by digestion with *Eco*RI and filled in using the enzyme Klenow. The expression system was the LacSwitch IPTG inducible mammalian expression system (Stratagene). The vector pOPRSvicat was digested with *Not*1, filled in with the enzyme Klenow, and the insert was blunt end ligated and screened for orientation. The resulting plasmid pNO4 was used to cotransform CHO-K1 cells along with the Lac-repressor-expressing vector p3′SS using the calcium phosphate precipitation method as described earlier (*19*, *20*). CHO cells were maintained in α MEM medium plus 10% FCS and selected by the use of 600 μg/mL G418 plus 500 μg/mL hygromycin. Resistant colonies were identified and individual colonies were repropagated until reaching a higher density. Two of these, Erk15 and Erk17 were analyzed further for effects on MAP kinase and Na^+/H^+ exchanger activity. Cells were routinely maintained without the presence of IPTG. To induce the dominant negative mutation, 5 mM IPTG was added for 15−18 h.

MAP Kinase Assays. To confirm that the mutant cells were reduced in MAP kinase activity we measured the MAP kinase activity of Erk15 and ERK17 cells. MAP kinase activity was analyzed by the in-gel kinase assay described earlier (*17*). For this purpose cells were grown in serum reduced medium (0.1%) overnight, then treated with 5 mM IPTG for 15−18 h. Cells were then stimulated with 10% serum for 2 or 5 min and harvested for IGKA assays with myelin basic protein as a substrate essentially as described earlier (*17*). The activity was quantified with a model BAS1000 phosphoimager.

Na^+/H^+ Exchanger Assays. To examine the effect of the reduction in MAP kinase activity of the mutant cell lines, we characterized intracellular pH regulation by the Na^+/H^+ exchanger. The fluorescent pH indicator 2′-7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used to monitor changes in cytosolic pH essentially as described earlier (*13*). Cells were cultured on glass coverslips and placed into a holder device and inserted into a 1 × 1 cm fluorescence cuvette at 37 °C and perfused with normal buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, and 10 mM Hepes-Tris pH 7.4). For this purpose cells were grown in serum reduced (0.1%) medium overnight and treated with 5 mM IPTG. In some experiments, intracellular acidosis was induced by exposing the cells at 37 °C to 15 mM NH_4Cl in normal buffer, for 5 min prior to its rapid withdrawal. They were then transferred to a Na^+ free buffer (135 mM *N*-methyl glucamine, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, and 10 mM Hepes-Tris, pH 7.4). pH recovery was obtained by transferring the cells to normal buffer at 37 °C. Fluorescence determinations were carried out using a Shimadzu RF5000U spectrofluorophotometer. The excitation wavelength was varied between 452 and 500 nm, and the emission wavelength remained at 520 nm. In some cases, cells were loaded with BCECF, allowed to equilibrate in normal buffer for 5 min and then transferred to a hypertonic buffer with a higher osmotic concentration consisting of normal buffer plus 100 mM additional NaCl. This procedure has been used earlier to osmotically activate the Na^+/H^+ exchanger (*21*). Statistical analysis of results of intracellular pH measurements was done using a Mann-Whitney U test.

RESULTS

To study the carboxyl-terminal of the Na^+/H^+ exchanger, we produced the carboxyl-terminal 343 amino acids as a fusion protein with GST. The product was purified using a two-step purification procedure. Figure 1 shows an SDS−PAGE of the purified GST-PCR343 protein. The upper band corresponds to the appropriate full length product. Despite the presence of protease inhibitors throughout the purification procedure, there was usually a small amount of degraded

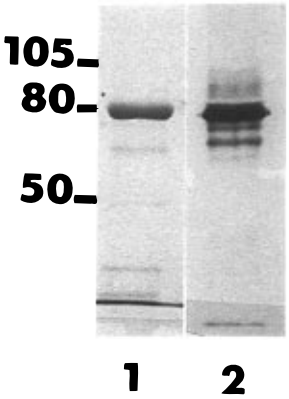

FIGURE 1: SDS−PAGE and immunoblot analysis of Na^+/H^+ exchanger fusion protein (PCR343). Na^+/H^+ exchanger, GST fusion protein (PCR343), was produced and purified as described in Materials and Methods. Lane 1 shows the purified product stained with Coomassie Blue. Lane 2 shows the results of immunoblotting with affinity purified antibody made against the carboxyl-terminal region of the protein. The positions and sizes of Bio-Rad prestained markers are as indicated.

product present. However, this made up only a very minor amount of the purified protein. To confirm the identity of the purified protein we used an antibody generated against the carboxyl-terminal of the Na^+/H^+ exchanger. This antibody was made against the carboxyl-terminal 178 amino acids of the Na^+/H^+ exchanger fused to β-galactosidase (*14*). The results of this Western blot analysis shows that the antibody reacted with the purified protein.

We used the purified PCR343 fusion protein to examine which kinases may be involved in regulation of the Na^+/H^+ exchanger. We isolated a soluble extract from mature rabbit skeletal muscle. The muscle extract was fractionated on a Mono Q column, and individual fractions were tested for their ability to phosphorylate purified PCR343. Figure 2 shows typical results of these studies. Purified PCR343 (lane 1) showed no auto phosphorylation in the presence of reaction mixture containing [γ-^{32}P]ATP. Unfractionated muscle extract (lane 2) and muscle extract with purified GST protein (lane 3) showed the presence of a minor band of M_r = 62 000. Addition of unfractionated muscle extract resulted in the appearance of a band of M_r = 66 000, the size of GST combined with the 343 carboxyl-terminal amino acids of the Na^+/H^+ exchanger (lane 4). Addition of the flow-through of the Mono Q column (unbound) also resulted in a small amount of phosphorylation of PCR343 (lane 5). When we examined the fractionated muscle extract, we found a reproducible pattern of phosphorylation of the Na^+/H^+ exchanger. Typical results are shown in lanes 6−36. There was an early peak of phosphorylation at fraction 8 followed by a broad peak of phosphorylation which had centered at fractions 14−20. To characterize this pattern of phosphorylation in more detail, we repeated the experiment several times measuring the amount of phosphorylation into the Na^+/H^+ exchanger containing band using a phosphoimager or scintillation counting of the excised band. The summary of these results is shown in Figure 3. There was an early narrow peak of phosphorylation centered around fraction 8 and a second broader peak of phosphorylation between fractions 14 and 21.

To examine which kinases might be involved in phosphorylation of the fusion protein, we used antibodies to immunoprecipitate MAP kinase from the skeletal muscle

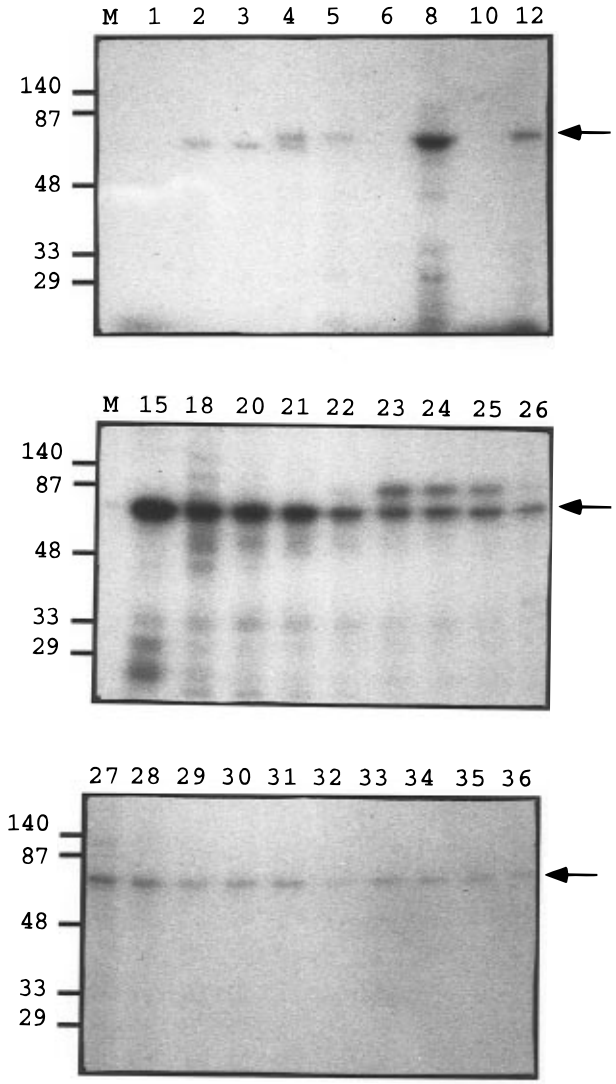

FIGURE 2: Phosphorylation of GST-Na^+/H^+ exchanger fusion protein by skeletal muscle extracts. The PCR343 fusion protein was expressed and purified as described in the Materials and Methods. Soluble skeletal muscle extracts were prepared and fractionated by FPLC chromatography using a Mono Q 10/10 column. Samples were incubated in a buffer containing [γ-^{32}P]ATP and muscle extracts and fusion proteins as follows: lane M markers used for size calibration, lane 1, PCR343 (250 ng); lane 2 unfractionated muscle extract; lane 3 muscle extract and GST protein alone; lane 4, unfractionated muscle extract and PCR343; lane 5 Mono Q 10 flow-through and PCR343; lanes 6−36 PCR343 (250 ng) and fractions 6−36, respectively. Arrow denotes the location of PCR343 of 66 kDa.

fractions. The results (Figure 4) show that MAP kinase was highly enriched in fractions 16−18. To determine if the kinase which phosphorylated the Na^+/H^+ exchanger was removed from the rabbit skeletal muscle fractions, we used the supernatant of the fractions immunoprecipitated by MAP kinase. We examined the phosphorylation of Na^+/H^+ exchanger fusion protein by these fractions. The results are shown in Figure 5. There was clearly a depletion of the phosphorylation originating from fractions 14 to 19. In addition there was no evidence of phosphorylation from fraction 8. Two narrow peaks of phosphorylation remained in fractions 19 and 21, which were earlier contained within the broad peak of fractions 14−21. In a similar experiment, we tried to immunodeplete fractions of protein kinase C using a commercial antibody against the protein. In this case, there

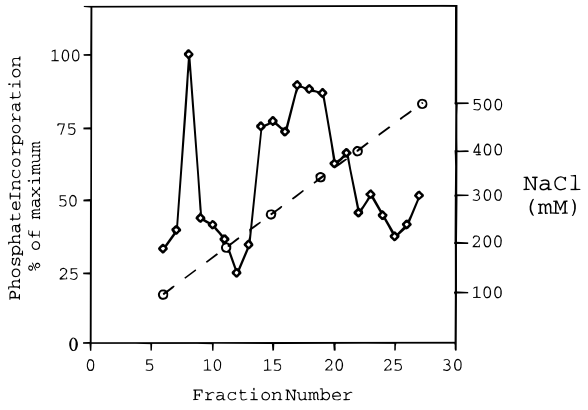

FIGURE 3: Summary of phosphate incorporation into GST-Na^+/H^+ exchanger fusion proteins by skeletal muscle extracts fractionated by FPLC chromatography. Skeletal muscle fractionation and phosphorylation studies were conducted as described for Figure 2. Samples were separated using SDS−PAGE and phosphorylated PCR343 protein was quantified by scintillation counting of excised bands or by analysis of incorporated radioactivity using a model BAS1000 phosphoimager (Fuji Photo Film Co., Ltd.). Results are typical of three independent experiments.

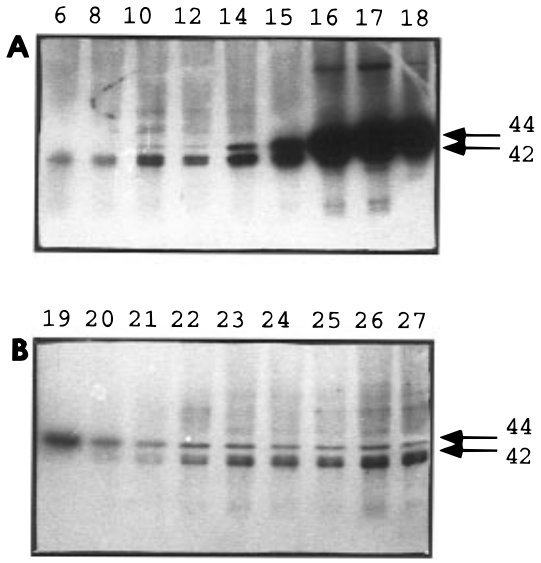

FIGURE 4: Immunoprecipitation of MAP kinase in rabbit skeletal muscle fractions. Solubilized muscle extracts were immunoprecipitated with anti-MAP kinase (Erk1-CT) antibody as described in the Materials and Methods. Immunoprecipitates were then run on 9% SDS−PAGE gels and immunoblotted with anti-MAP kinase antibody. Lane numbers correspond to fraction numbers. (A) Fractions 6−18 and (B) fractions 19−27.

was no significant change in the shape of the phosphorylation profile (not shown).

To examine the stoichiometry of the phosphorylation of the Na^+/H^+ exchanger fusion protein, we used different size carboxyl-terminal antiporter fusion proteins. Typical results are shown in Figure 6. Purified MAP kinase phosphorylated the Na^+/H^+ exchanger fusion proteins similar to that seen with skeletal muscle fractions. The stoichiometry of phosphorylation was 1.04 ± 0.03 mol of Pi/mol of PCR343. Similar experiments with two other Na^+/H^+ exchanger fusion proteins, PCR 272 and PCR 178 showed that they contained 0.40 and 0.87 mol of phosphate, respectively. These results indicate that the site of phosphorylation by MAP kinase is in the terminal 178 amino acids of the Na^+/H^+ exchanger. The reason for the lower level of phosphorylation of PCR

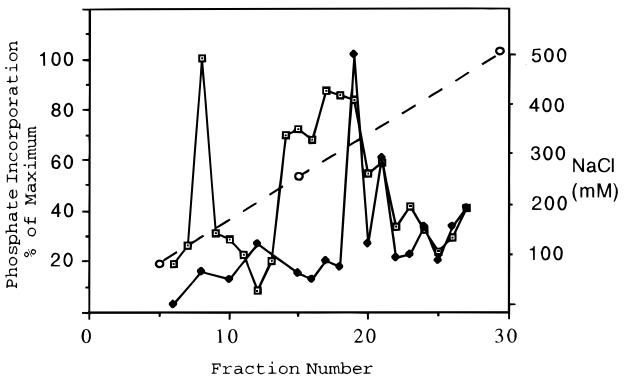

FIGURE 5: Phosphorylation of GST-Na^+/H^+ exchanger fusion protein by skeletal muscle extracts. The PCR343 fusion protein was expressed and purified as described in the Materials and Methods. Soluble skeletal muscle extracts were prepared and fractionated by FPLC chromatography using a Mono Q 10/10 column. (⊡) Phosphorylation by control muscle extracts. (♦) Phosphorylation by extracts in which MAP was removed by immunoprecipitation.

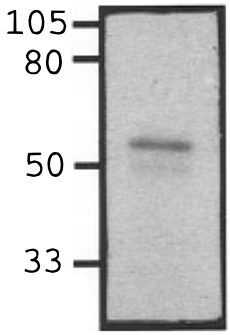

FIGURE 6: Phosphorylation of GST-Na^+/H^+ exchanger fusion protein (PCR272) by purified MAP kinase. Purified GST-Na^+/H^+ exchanger fusion protein was phosphorylated by purified MAP kinase as described in the Materials and Methods. The reaction was started by addition of ATP and terminated by adding gel sample buffer for SDS−PAGE and autoradiography.

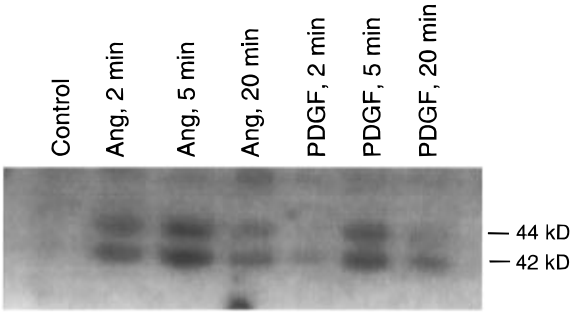

FIGURE 7: Time course for activation of MAP kinase-dependent phosphorylation of the carboxyl-terminal region of the Na^+/H^+ exchanger (PCR343). Growth arrested WKY vascular smooth muscle cells were stimulated with 100 nmol/L Ang II or 10 ng/mL PDGF for the indicated times. Cells were harvested in lysis buffer as described in Materials and Methods and in-gel kinases assays were performed using cytosolic extracts and GST-Na^+/H^+ exchanger fusion protein incorporated into the gel.

272 is not known but could be due to the particular conformation of this protein.

We used another independent system to confirm that MAP kinase-dependent phosphorylation of the cytoplasmic domain could occur. In this system we used an IGKA to examine phosphorylation of the PCR343 protein by cell extracts of smooth muscle cells of WKY rats. The results are shown in Figure 7. Cell extracts of WKY rats showed MAP kinase

phosphorylation and phosphotransferase activity, particularly when stimulated with either angiotension II or PDGF. Controls with either no substrate or GST alone showed no indication of phosphorylation (not shown). These results show that MAP kinase phosphotransferase activity directed toward the carboxyl-terminal of the Na^+/H^+ exchanger exists in extracts from smooth muscle of WKY rats and can be stimulated hormonally.

To examine the effect of MAP kinase on Na^+/H^+ exchanger activity, we constructed an inducible MAP kinase dominant negative mutant as described in Materials and Methods. CHO cells were used because they have been well characterized with regard to MAP kinase activity. In addition, myogenic cell lines were avoided because they exhibit a number of changes in cell character including differentiation when subjected to serum withdrawal (*20*). A number of mutant CHO cell lines were made, Erk15 and Erk17 were chosen for further analysis. The activity of MAP kinase in these mutants was measured in cells grown in serum reduced medium for 24 h or in cells stimulated with 10% serum for either 2 or 5 min. In all cases, the activity of the mutants was reduced in comparison to that of controls. For the Erk15 mutant, the activity was reduced to 68, 56, and 32% of the values of control cells with 0, 2, and 5 min of serum stimulation, respectively. For the Erk17 mutant, the activity was 30, 66, and 74% of the control cells with 0, 2, and 5 min of serum stimulation, respectively. The results were mean of three experiments.

We then tested the effect of MAP kinase on the activity of the Na^+/H^+ exchanger. We compared the antiporter activity in CHO cells and the mutants Erk15 and Erk17. Cells were maintained in serum reduced medium overnight in the presence of IPTG. Intracellular pH was measured as described above. Figure 8A shows typical effects of addition of serum on resting intracellular pH of CHO and Erk15 cells. Addition of serum raised resting intracellular pH in a slow but steady manner. The mutant Erk15 also responded in a similar manner, but the resting intracellular pH only increased by about half as much. HMA totally abolished the effect of serum addition showing that the effect was due to activity of the Na^+/H^+ exchanger. A summary of a series of experiments for CHO, Erk15, and Erk17 cells is shown in Figure 8B. Both Erk15 and Erk17 cells had a diminished response to serum addition in comparison to CHO cells. HMA addition nearly eliminated the effect of serum addition in all the cell types.

We also tested the effect of hypertonic stimulation of the Na^+/H^+ exchanger in the three cell types. Typical results are shown in Figure 9. There was no significant difference between CHO and Erk15 cells in hypertonic stimulation of the Na^+/H^+ exchanger (Figure 9). HMA completely eliminated the effect of hypertonic stimulation in both Erk15 and CHO cells. Similar results were obtained for Erk17 cells (not shown).

In one set of experiments, we examined the ability of CHO, Erk15, and Erk17 cells to recover from an acute acid load induced by ammonium chloride. We found that there was no significant difference between these cells' abilities to recover from an acid load when they recovered from an acid load in medium containing 135 mM NaCl (not shown).

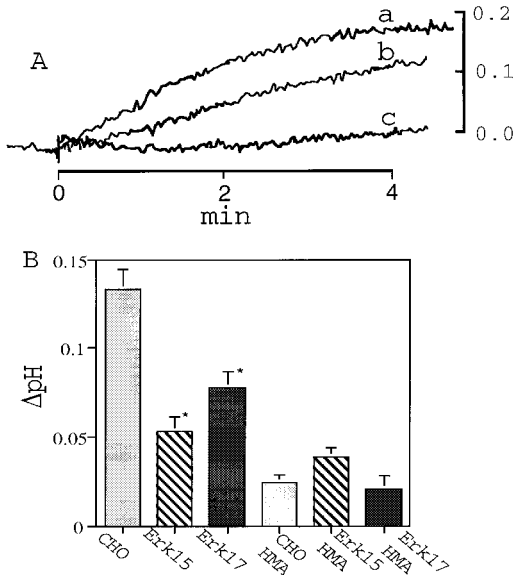

FIGURE 8: Effect of serum on steady state intracellular pH of control CHO cells and mutants erk15 and Erk17. Cells were grown on coverslips and maintained as described in Materials and Methods. CHO, Erk15, and Erk17 cells were treated overnight with low serum (0.1%) and 5 mM IPTG. (A) Typical effects of serum addition on intracellular pH. Trace a, CHO cells, b, Erk 15 cells, c, CHO with 1 uM HMA present. (B) Summary of effects of addition of 10% serum on resting intracellular pH. Serum was added to resting cells and the change from resting intracellular pH was measured two min after addition. Results are mean ±SE of at least 10 experiments. (*) Denoted values are significantly different from CHO values at $P < 0.001$.

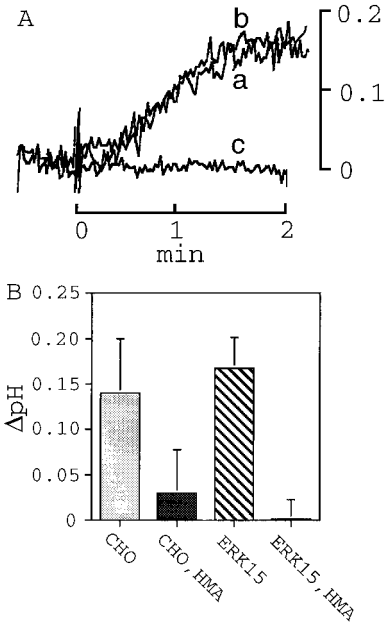

FIGURE 9: Hypertonic activation of the Na^+/H^+ exchanger in CHO and erk15 cells. Cells were prepared as in Figure 7 except all cells received normal medium containing serum. (A) Where indicated, the medium was made hypertonic by an extra 100 mM NaCl addition. Trace a, CHO cells, b, Erk 15 cells, c, CHO cells with 1 uM HMA present. (B) Summary of effects of hypertonic stimulation on CHO and Erk15 cells. Results are mean ±SE of at least seven experiments.

DISCUSSION

The regulation of Na^+/H^+ exchanger activity by phosphorylation has been the subject of intense investigation. It has been shown that growth factors induce phosphorylation of

the protein and that the phosphorylation is in the C-terminal 179 amino acids of the protein (*5*, *6*). Growth factor activation results in activation of the protein, and recently, it was suggested that increased Na^+/H^+ exchanger activity in primary hypertension is associated with increased phosphorylation of NHE1 (*22*). Not all of NHE1 activation is mediated by direct phosphorylation, and removal of the carboxyl-terminal 179 amino acids still allows up to 50% of growth factor activation. However, phosphorylation certainly results in a significant part of activation of the protein (*23*).

In this study, we examine the role of MAP kinases in phosphorylation and regulation of activity of the Na^+/H^+ exchanger. MAP kinases are known to be activated by the same type of extracellular stimuli that also activate the Na^+/H^+ exchanger including growth factors and hormones. In initial experiments, we fractionated extracts from rabbit skeletal muscle and found that some specific fractions were able to phosphorylate a Na^+/H^+ exchanger-GST fusion protein of the cytoplasmic domain of the antiporter (Figure 2). GST alone was not phosphorylated by the fractions. We showed that several of the fractions were enriched in MAP kinase (Figure 4) and that removal of the MAP kinase protein resulted in a reduction in the ability of some specific fractions to phosphorylate the carboxyl-terminal of the Na^+/H^+ exchanger. This led us to suspect that MAP kinases were involved in regulation of the protein. It was not possible to demonstrate directly from these experiments that MAP kinase phosphorylates the Na^+/H^+ exchanger. It is possible that some other associated kinase or another kinase in the same fraction was affected by MAP kinase and was responsible for the actual phosphorylation.

To examine if MAP kinase could directly phosphorylate the carboxyl-terminal of the exchanger we used purified MAP kinase protein. The stoichiometry of phosphorylation was only 1 mol of phosphate/mol of protein. This amount, though significant, could likely account for only a part of the phosphorylation reported to occur on the cytoplasmic domain (*5*, *6*). It is clear that other kinases must also be involved in regulation of this region. We note, however, that immunoprecipitation of MAP kinase removed a large majority of the phosphorylation by several different fractions (Figure 5). The reason for this is not yet known, however, it may be that MAP kinase was responsible for activation of these kinases in those fractions. An alternative explanation is that other kinases involved in phosphorylation of the exchanger are inactive in this assay system. It is possible that the association of subunits of some kinases did not survive fractionation by FPLC, and therefore, their activity is not shown by this analysis. It was interesting that two peaks of phosphorylation remain intact even after immunoprecipitation of MAP kinase. The identity of the kinases in these fractions is not known and awaits further experimentation.

We also examined MAP kinase-dependent phosphorylation of the carboxyl-terminal region of the protein by cytosolic fractions of vascular smooth muscle cells from WKY rats. Once again, this independent method confirmed that MAP kinase could phosphorylate this region of NHE1. The results were also very interesting in that hormonal stimulation of quiescent WKY smooth muscle cells by either Ang II or PDGF markedly enhanced phosphorylation of the Na^+/H^+ exchanger fusion protein by both $p42^{mapk}$ and $p44^{mapk}$. These results are consistent with a hormonal activation being mediated, at least partially, by MAP kinase-dependent phosphorylation of the protein. A recent report (*24*) has suggested that MAP kinase may not directly phosphorylate the Na^+/H^+ exchanger. The reason for the discrepancy between these results and the present study is not clear. Our study used the rabbit NHE1 protein, which varies slightly in sequence from the human protein. In addition, it may be that the relatively low level of phosphorylation we observed (1 mol/mol of protein) was not detectable in this *in vitro* system (*24*). In addition, we observed what may be a structural dependence of phosphorylation. The C-terminal fusion proteins of 343 and 178 amino acids showed double the level of phosphorylation of the fusion protein of 272 amino acids. This suggests that the conformation of the protein may affect accessibility to the kinase. It may be that the detergent solubilized protein with the hydrophobic membrane domain is not accessible to phosphorylation. In the present study, we showed phosphorylation of the C-terminal both *in vitro* for the soluble carboxyl-terminal region and in an IGKA with immobilized protein. In addition, in the IGKA the phosphorylation was stimulated by hormonal activation, which suggests an important physiological role for this form of regulation.

It has been suggested earlier that MAP kinase is involved in hormonal activation of the Na^+/H^+ antiporter (*25*). We therefore attempted to examine the role of MAP kinases in regulation of NHE1 activity. To do this, we made an inducible dominant negative MAP kinase mutant. Attempts at constructing permanently defective MAP kinase cells were not successful, likely due to the critical function of these proteins in cell growth and proliferation. We found that we could induce the mutant cells to have defective MAP kinase by overnight treatments; however, longer term induction caused the cells to be unable to proliferate (not shown). When we examined the efficacy of serum to stimulate the Erk mutant cells, we found that activation of the antiporter in mutant cells was reduced greatly. In control cells, resting intracellular pH routine rose 0.12−0.15 pH units, while in the mutants, the effect was reduced 50−70% (Figure 8). These results confirm that MAP kinases have an important physiological effect on the Na^+/H^+ exchanger.

It was possible that we had caused a number of nonspecific alterations to the cell which indirectly affected the Na^+/H^+ exchanger. Therefore, we examined some other methods of activation of the protein. Hypertonic stimulation of the exchanger is one method in which to activate the protein in a phosphorylation independent manner in a pathway independent of MAP kinase activation (*21*, *24*). The mechanism of activation may be through a region of the protein proximal to amino acid residue 698 (*26*). We tested control and MAP kinase mutants and found no significant effect on this different mechanism of activation of the antiporter in the control and Erk15 cells (Figure 9). In addition, we found that there was no difference in activation of the antiporter by an acid load in these cells. These results suggest that the effect we saw on activity of the protein was specific to hormonal regulation of the protein and not due to some general defect induced in either the cells or the protein itself.

Overall, our results suggest that MAP kinase can phosphorylate the carboxyl-terminal of the Na^+/H^+ exchanger and affect the activity of the protein. The level of phosphorylation was only 1 mol of phosphate/mol of protein.

Clearly, this type of regulation is not the only type of phosphorylation involved *in vivo*. However, it appears to be a significant aspect of regulation of the Na^+/H^+ antiporter by phosphorylation. In our experiments, we noted that this phosphorylation was stimulated by PDGF and AngII. This supports the hypothesis that MAP kinase-mediated phosphorylation of the exchanger is hormonally regulated. Our results are at variance with those of Aharonovitz and Granot (*27*) who suggested that MAP kinase activation of the exchanger is through a phosphorylation-independent pathway. However, their experiments were with human platelets which may regulate Na^+/H^+ exchanger activity differently than muscle cells. It has been suggested that regulation of activity of the exchanger can vary greatly between cell types, even within the same isoform of the protein (*28*). In this study, we primarily examined muscle cell lines. In several types of muscle, MAP kinase-dependent pathways are thought to play an important role in cell growth and proliferation in muscle and other cell types (*17*, *29*, *30*). In addition, activation of the Na^+/H^+ exchanger is also thought to be important in these processes in many cell types (*1*, *29*, *31*). Our studies suggest that MAP kinase mediated phosphorylation of the protein is important in mitogenic activation in some cell types. However, this does not exclude an addition role for a protein mediator of MAP kinase in other cell types or even in the same cells (*9*, *10*, *24*). Future studies will examine how MAP kinase activation of the Na^+/H^+ exchanger occurs in a number of other tissues. In addition the role of regulation by other protein kinases of the antiporter has yet to be resolved.

ACKNOWLEDGMENT

L. F. is a Senior Scholar of the Alberta Heritage Foundation for Medical Research. S.P. is a Medical Research Council of Canada Industrial Scientist. We thank Dr. B.C. Berk (University of Washington) for his assistance with the IGKA.

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BI970802F