Role of protein kinase C and transcription factor AP-1 in the acid-induced increase in Na/H antiporter activity

(*c-fos/c-jun/sphingosine/memory*)

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ABSTRACT Chronic incubation of cultured renal tubular epithelial cells in acid medium causes an increase in Na/H antiporter activity that persists after removal from acid, is dependent on protein synthesis, and is associated with an increase in Na/H antiporter mRNA. Chronic activation of protein kinase C has similar effects in these cells. The present studies examined the role of protein kinase C in the effect of acid incubation. Incubation of MCT cells in acid for 24 h caused a 50% increase in Na/H antiporter activity. This was prevented by inhibition of protein kinase C, either with sphingosine or by protein kinase C downregulation. Pertussis toxin pretreatment did not prevent the increase in antiporter activity. Acid incubation caused an increase in transcription factor AP-1 activity, as shown by an increase in expression from a reporter gene containing six tandem AP-1 binding sites. This was associated with transient increases in *c-fos* and *c-jun* mRNAs. This response is typical of that for gene activation by protein kinase C. These studies demonstrate that acid activation of the Na/H antiporter requires protein kinase C and is associated with *c-fos* and *c-jun* expression and increased AP-1 activity.

Chronic acidosis causes an increase in renal proximal tubular Na/H antiporter and Na/3HCO₃ transporter activities that persists after the transporters are removed from the acidotic milieu (1–5). The effect on the Na/H antiporter can be simulated by incubation of a rabbit primary proximal tubule cell culture, or a number of renal cell lines, in acid medium for 24–48 h (6–8). This effect of acid incubation is dependent on protein synthesis and is associated with an increase in Na/H antiporter mRNA (mRNA_{Na/H}) abundance at 24 h (6–8). In rabbit proximal tubule primary cultures, chronic activation of protein kinase C (phorbol esters for 2 h) causes an increase in Na/H antiporter activity that persists 24 h after removal of phorbol ester, is dependent upon transcription and translation, and is associated with a 2-fold increase in mRNA_{Na/H} (9). This response to chronic activation of protein kinase C is similar to the response to chronic acid incubation. The present studies examined whether protein kinase C mediates the chronic activation of the Na/H antiporter by acid.

MATERIALS AND METHODS

Cell Culture. MCT cells (a simian virus 40-transformed mouse proximal tubule cell line) were grown in RPMI medium with 5% (vol/vol) fetal bovine serum and subcultured upon reaching confluence. For experimentation, MCT cells were subcultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) (pen/

strep), and 5% fetal bovine serum, grown to confluence, and serum-deprived for 48 h prior to and during experimental incubations. Acid medium was prepared by titrating control medium with sufficient HCl to decrease the HCO₃ concentration by 50% (6).

Measurement of Na/H Antiporter Activity. To assay Na/H antiporter activity, MCT cells were grown on glass coverslips and loaded with the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), by incubation in the acetoxymethyl ester of BCECF (10 µM) for 40 min at 37°C. After washing, the coverslip was placed in a plastic cuvette in a computer-controlled spectrofluorimeter (SLM 8000C). Cell pH was estimated from the ratio of fluorescence with excitation at 500 nm and 450 nm and emission at 530 nm, as described (6, 10).

Na/H antiporter activity was assayed as the initial rate of Na-dependent cell pH increase after an acid load by a NH₃/NH₄⁺ prepulse in the absence of CO₂/HCO₃ (6). For this assay, the Hepes-buffered standard sodium solution contained 138 mM Na, 4.5 mM K, 1.1 mM Ca, 1.5 mM Mg, 147.7 mM Cl, and 15 mM Hepes. In Na-free solutions, Na was replaced with choline. In Na-free NH₃/NH₄⁺ solutions, 20 mM ammonium replaced 20 mM choline. All solutions were adjusted to pH 7.4 with *N*-methyl-D-glucammonium hydroxide at 37°C. Cells were first perfused with the Na-containing solution and then perfused with the Na-free NH₃/NH₄⁺ solution for 5 min. Perfusate was then changed to the Na-free solution, which caused the cell pH to decrease and reach a stable plateau. Subsequently, Na was added, causing cell alkalization; the initial rate of alkalization was used as an index of Na/H antiporter activity, as described (6). In all experiments reported, buffer capacity, measured as described from the cell pH response to NH₃/NH₄⁺ removal (6, 11), was unaffected by the experimental maneuver.

RNA Blots. RNA was prepared using a modification of the method of Chirgwin (7, 12). Confluent cell monolayers were homogenized in guanidinium thiocyanate, centrifuged through a CsCl cushion, further purified by phenol/chloroform extraction and ethanol precipitation, and poly(A)⁺-selected by oligo(dT) chromatography. Poly(A)⁺ RNA samples were fractionated by formaldehyde/agarose gel electrophoresis and transferred to nylon membranes. Templates used for probe synthesis were excised from the appropriate plasmids with restriction enzymes and purified by agarose gel electrophore-

Abbreviations: CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; G protein, guanine nucleotide binding protein; EIPA, ethylisopropylamiloride; mRNA_{Na/H}, Na/H antiporter mRNA.

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sis. For the chloramphenicol acetyltransferase (CAT) probe, a 550-base-pair *Xho*I–*Nco*I insert was excised from pBLCAT3 (13). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.1-kilobase *Bam*HI–*Hind*III insert was used (14). For *c-fos* and *c-jun*, 2.2- and 2.6-kilobase full-length cDNA inserts were used, respectively (15, 16). Nucleic acid probes were synthesized to specific activities of $>5 \times 10^8$ cpm/ μ g by DNA polymerase I (Klenow fragment) with random hexanucleotide priming and purified with NEN sorb (DuPont/NEN). Nylon filters were prehybridized in $5 \times$ SSC (0.75 M NaCl/75 mM trisodium citrate)/5 $\times$ Denhardt's solution [Ficoll (1 mg/ml)/polyvinylpyrrolidone (1 mg/ml)/bovine serum albumin (1 mg/ml)]/0.5% SDS/50% (vol/vol) formamide/denatured salmon testis DNA (0.5 mg/ml) at 42°C. Boiled probes were then added for hybridization under the same conditions. Filters were then sequentially washed in $1 \times$ SSC/0.1% SDS at 20°C for 15 min and $0.1 \times$ SSC/1% SDS at 55°C for 1 h and exposed to Kodak X-Omat film at –70°C with intensifying screens. Signals on autoradiographs were quantified by laser densitometry (7). GAPDH transcript levels served as loading controls.

Cell Transfection/CAT Measurements. MCT cells were cotransfected with either p6xTRE/IFNCAT [a CAT reporter plasmid with six AP-1 binding sites in tandem upstream of a minimal interferon promoter (17)] or pBLCAT2 [a CAT reporter plasmid with a thymidine kinase promoter (13)], and pSV2-Neo using Lipofectin reagent (BRL). Populations of cells were selected with G418 (RPMI medium containing 10% fetal bovine serum and G418 at 400 μ g/ml) and screened for CAT enzyme and message expression in response to phorbol ester. Pools of G418-resistant clones were used to be certain that the site of insertion in a single clone did not significantly affect the results. For studies, cells were plated in the same medium for 2 days and then changed to DMEM/Ham's F12 with pen/strep for 3 days prior to experimentation.

After an experimental maneuver, cells were washed, scraped, and pelleted. The pellet was resuspended in 100 μ l of 250 mM Tris-HCl (pH 7.8) and disrupted by a freeze-thaw cycle. The debris was pelleted, and the supernatant used for assays. In each CAT reaction mixture, a volume of cell extract corresponding to 100 μ g of protein (Bio-Rad protein assay) was incubated with a mixture of 1 μ l of [14 C]chloramphenicol (56.2 mCi/mmol, 25 μ Ci/ml; 1 Ci = 37 GBq), 20 μ l of 4.0 mM acetyl coenzyme A, and 70 μ l of 1.0 M Tris-HCl (pH 7.8; total volume, 150 μ l) at 37°C for 50 min, and the reaction was stopped by extraction with 1 ml of ethyl acetate. The supernatant was evaporated, and the pellet was resuspended in 10 μ l of ethyl acetate and spotted on a silica gel 1B thin layer chromatography plate (J. T. Baker). Acetylated and nonacetylated chloramphenicol were separated with chloroform/methanol, 19:1 (vol/vol). The thin layer chromatography plate was air-dried and exposed to x-ray film. CAT assays were quantitated by scraping the spots from the plates and measuring the radioactivity in the spots in a liquid scintillation counter. Untransfected cells had no CAT activity.

Chemicals. All chemicals were obtained from Sigma unless otherwise noted as follows: penicillin and streptomycin from Whitaker Bioproducts; culture media from GIBCO Laboratories Life Technology; 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein from Molecular Probes; phorbol 12-myristate 13-acetate (PMA) from LC Services (Woburn, MA); and [14 C]chloramphenicol and [32 P]dATP from New England Nuclear.

RESULTS

Inhibition of Protein Kinase C Prevents the Acid-Incubation-Induced Increase in Na/H Antiporter Activity. If protein kinase C plays a key role in mediating the effect of acidosis

FIG. 1. Effect of sphingosine on the acid-induced increase in Na/H antiporter activity. MCT cells were incubated in control or acid medium for 24 h and then removed from the incubation medium, and Na/H antiporter activity was assayed. To inhibit protein kinase C, plates were incubated in 10 μ M sphingosine plus 10 μ M albumin for 12 h prior to and then during the entire control or acid incubation. As a control, cells were incubated for a similar time in vehicle (10 μ M albumin) alone. The y axis is the initial rate of increase in cell pH in response to Na addition (dpHi/dt). NS, not significantly different.

on the Na/H antiporter, then inhibition of protein kinase C should prevent this response to acid. Protein kinase C was inhibited in two ways. First, we used sphingosine (18). Cells were incubated in 10 μ M sphingosine plus 10 μ M albumin for 12 h prior to exposure to control or acid medium and then throughout 24 h of control or acid incubation (19). Fig. 1 shows the results. In control cells incubated in albumin alone, the 24-h acid incubation caused a 47% increase in Na/H antiporter activity. However, in sphingosine-treated cells, acid incubation had no effect on Na/H antiporter activity.

Our second approach was to downregulate protein kinase C by pretreatment with high concentrations of PMA (20). Cells were pretreated with 400 nM PMA for 48 h prior to and during control or acid incubation (24 h). As shown in Fig. 2, in cells not pretreated with PMA, a 24-h acid incubation caused a 46% increase in Na/H antiporter activity. In PMA-pretreated cells, acid incubation had no effect on Na/H antiporter activity. There was a small increase (19%) in Na/H antiporter activity secondary to PMA treatment (control vs.

FIG. 2. Effect of PMA pretreatment on the acid-induced increase in Na/H antiporter activity. Protocol is similar to that of Fig. 1, except that in these studies protein kinase C was inhibited by pretreating cells with 400 nM PMA for 48 h prior to and during incubation in control or acid medium. NS, not significantly different.

FIG. 3. Effect of acid incubation or PMA on AP-1 activity. MCT cells were transfected with reporter plasmid p6xTRE/IFNCAT and exposed to control medium (lanes c), acid medium (lanes a), or PMA (lanes p) for the times indicated. Shown is an autoradiograph of a thin layer chromatography plate.

PMA pretreatment control). This may reflect activation of small amounts of synthesized protein kinase C with secondary increases in antiporter activity (9).

The above results suggest an important role of protein kinase C in acid-induced activation of the Na/H antiporter. Activation of protein kinase C by hormones can be independent of or dependent on pertussis toxin-sensitive guanine nucleotide binding proteins (G proteins). To test whether activation of the Na/H antiporter by acid was mediated by a pertussis toxin-sensitive G protein, MCT cells were pretreated for 4 h with pertussis toxin (200 ng/ml). This treatment eliminates >90% of subsequent pertussis toxin-induced [32 P]ADP labeling of cell membranes. In pertussis toxin-pretreated cells, acid incubation caused a 28% increase in Na/H antiporter activity [1.71 ± 0.04 pH units/min (acid) vs. 1.34 ± 0.12 (control); $P < 0.05$]. Thus, the effect of acid does not require pertussis toxin-sensitive G proteins. It is possible that other G proteins that are pertussis toxin-insensitive but able to activate protein kinase C, such as G_{α_q} possibly, are mediating this effect (21).

Acid Incubation Increases AP-1 Activity. To examine whether acid incubation chronically increases protein kinase C in a manner capable of gene activation, we examined the effect of acid incubation on AP-1 activity. Many protein kinase C-responsive genes possess a consensus nucleotide sequence in their 5' flanking region that binds the nuclear factor AP-1 and is responsible for protein kinase C induction of the gene (22). To test whether acidosis caused an increase in AP-1 activity in renal cells, MCT cells were stably trans-

FIG. 4. Effect of acid or PMA on CAT mRNA. Studies are similar to that performed in Fig. 3, except that CAT mRNA abundance was measured. Multiple CAT transcripts were present, probably due to multiple sites of initiation. A transcript of 1640 nucleotides (nt), the appropriate size for initiation from the transfected interferon promoter, is indicated by the arrow. Results with the GAPDH probe are shown as a control.

FIG. 5. Effect of acid incubation on c-fos mRNA. At time 0, medium was exchanged to either control medium (lanes c) or acid medium (lanes a). Results with the GAPDH probe are shown as a control.

fected with a construct containing six AP-1 binding sites, a minimal interferon promoter, and a CAT reporter gene (17). Cells were incubated in control medium (pH 7.3), acid medium (pH 7.0), or control medium plus 100 nM PMA. In all groups, medium was exchanged at time 0. Both acid incubation and PMA increased CAT activity 3- to 4-fold at 3 h, an increase that persisted for 24 h (Fig. 3). In MCT cells stably transfected with a CAT reporter gene driven by a thymidine kinase promoter, acid incubation had no effect on CAT activity. Thus, acid incubation is not a generalized stimulator of transcription.

To ensure that the increase in CAT activity was not due to an effect on translation, we measured CAT mRNA abundance by Northern blot analysis. As shown in Fig. 4, transfected MCT cells possessed CAT mRNA of multiple sizes. This may be due to false initiation from neighboring plasmid or host sequences. Transcription from the interferon promoter predicts an mRNA of 1640 nucleotides. Abundance of this mRNA was increased by 100 nM PMA at 1 and 3 h. Acid incubation had no effect on CAT mRNA at 1 h but caused a 2.5-fold increase at 3 h.

Acid Incubation Increases c-fos and c-jun mRNA Abundance. AP-1 activity is potently expressed by a c-fos-c-jun dimer. Therefore, we next examined the effect of acid incubation on c-fos and c-jun mRNA. mRNA levels in acid-incubated cells were compared to levels in control cells that underwent a fluid exchange to control medium. As shown in Fig. 5, acid incubation caused c-fos mRNA to increase 2.5-fold at 15–30 min and then to return rapidly to control levels. A similar response was seen in three experiments. In two additional experiments, fluid exchange caused a large increase in c-fos mRNA abundance. In these experiments, no additional effect of acid could be demonstrated.

Fig. 6 shows results with c-jun mRNA. There were two c-jun transcripts, as reported (16, 23), and both were present in control cells. Acid incubation caused c-jun mRNA to increase 2-fold at 1 and 2 h and then to return to control levels. No increase in c-jun mRNA was noted prior to 1 h. This time course with c-jun mRNA increasing later than c-fos mRNA

FIG. 6. Effect of acid incubation on c-jun mRNA. At time 0, fluid was exchanged to control medium (lanes c) or acid medium (lanes a). Results with the GAPDH probe are shown as a control.

and persisting for longer times is typical of protein kinase C activation and may be due to fos-induced repression of c-fos- and AP-1-induced activation of c-jun (24, 25). Thus, these studies demonstrate that incubation of MCT cells in acid medium causes expression of c-fos and c-jun, leading to increased AP-1 activity and potentially activating a number of protein kinase C-responsive genes.

DISCUSSION

Incubation of cultured renal proximal tubule cells in acid medium for 48 h causes an increase in Na/H antiporter activity that persists after the cells are removed from the acid environment (6). This effect is seen whether medium pH is acidified by lowering the concentration of HCO₃ or increasing Pco₂ and is blocked by cycloheximide (6). Acid-induced stimulation of the Na/H antiporter is also seen in three renal cell lines with characteristics of the proximal tubule (MCT, OKP, and LLC-PK₁) but is not seen in cultured human foreskin fibroblasts or in NIH 3T3 cells (6–8). In MCT and LLC-PK₁ cells, acid incubation causes an increase in the abundance of mRNA_{Na/H} (7, 8).

In cultured proximal tubule cells, chronic activation of protein kinase C has an effect similar to that of chronic acid incubation: an increase in Na/H antiporter activity that is dependent on transcription and translation and is associated with increased abundance of mRNA_{Na/H} (9). In the present studies, we showed that the effect of acidosis on Na/H antiporter activity was blocked by inhibition of protein kinase C. Whereas sphingosine and PMA pretreatment may have nonspecific effects on cells, results of these studies were similar, suggesting that they were due to protein kinase C inhibition. These studies, thus, suggest that chronic activation of protein kinase C plays a key role in the response of the Na/H antiporter to chronic acidosis in kidney tubular cells.

Na/H antiporters have been categorized into two groups based on their sensitivity to amiloride analogs and their location in epithelial cells. Housekeeping Na/H antiporters are sensitive to nanomolar concentrations of ethylisopropyl-amiloride (EIPA) and are present in nonpolar cells and on the basolateral membrane of certain epithelial cells (26). A second Na/H antiporter requires micromolar concentrations of EIPA for inhibition and is present on the apical membrane of epithelial cells (26). The Na/H antiporter clone NHE-1 (27) corresponds to the EIPA-sensitive housekeeping antiporter. Acid incubation increases both types of Na/H antiporter in renal epithelial cells (7, 8). In MCT cells, the Na/H antiporter is EIPA-sensitive and mRNA encoding NHE-1 is regulated by acid. The present results, therefore, demonstrate a requirement for protein kinase C in acid activation of NHE-1. It is not presently clear whether acid activation of the EIPA-resistant Na/H antiporter also requires protein kinase C.

Acid incubation was demonstrated to increase AP-1 activity. The increase in AP-1 activity was preceded by an increase in c-fos and c-jun mRNA abundance. c-fos and c-jun are immediate early response genes (22). These genes are activated by serum, phorbol esters, and a number of growth factors (including epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor) and are believed to play a key role in cell growth. In addition, c-fos and c-jun contribute to regulation of a number of processes other than cell growth: arabinofuranosylcytosine-, 1,25-dihydroxyvitamin D₃-, and protein kinase C-induced differentiation in myeloid cells (28, 29); tumor necrosis factor α stimulation of collagenase expression in fibroblasts (30); and α - and β -adrenergic- and protein kinase C-induced expression of myosin light chain 2 in neonatal myocardial cells (31). Interestingly, protein kinase C-induced myeloid differentiation is also associated with increases in Na/H antiporter mRNA abundance and transcription rate (32). c-fos is also activated by

cell depolarization in PC12 pheochromocytoma cells (33), and c-fos and c-jun are activated by transforming growth factor β in a number of cell types (34). The present studies now suggest an important role for c-fos and c-jun in acid-induced regulation of the renal Na/H antiporter. Interestingly, *in vivo* chronic metabolic acidosis is associated with renal proximal tubular hypertrophy, which may also be mediated by c-fos and c-jun (35).

In summary, chronic acidosis likely activates protein kinase C, secondarily leading to activation of the genes for c-fos and c-jun at 15–30 min and 1–2 h, respectively. This then leads to an increase in AP-1 activity, evident at 3 h. It is not presently clear whether acidosis directly activates protein kinase C or whether phospholipases C or D are activated. Although protein kinase C plays an important role in Na/H antiporter activation, the role of AP-1 requires further study. It is possible that (i) AP-1 directly activates the Na/H antiporter gene, (ii) AP-1 activates a second series of genes whose products are responsible for activation of the Na/H antiporter, or (iii) AP-1 is irrelevant to Na/H antiporter activation and other protein kinase C-dependent pathways mediate this effect (36, 37). In this regard, it should be noted that the acid-induced increase in mRNA_{Na/H} has not been demonstrated to be transcriptionally mediated. However, the 5' flanking region of the human Na/H antiporter gene (NHE-1) contains three AP-1 binding sites (38). One of these AP-1 sites is conserved in the rabbit Na/H antiporter gene (39).

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