Long-term activation of protein kinase c causes chronic Na/H antiporter stimulation in cultured proximal tubule cells.

S Horie, O Moe, R T Miller, R J Alpern

J Clin Invest. 1992;89(2):365-372. https://doi.org/10.1172/JCI115594.

Research Article

To examine the role of protein kinase C as a chronic regulator of proximal tubule Na/H antiporter activity, the effect of phorbol 12-myristate 13-acetate (PMA) on the Na/H antiporter was studied in cultured proximal tubule cells. Short-term activation of protein kinase C by 5 min exposure to PMA caused an acute increase in Na/H antiporter activity that was not prevented by cycloheximide or actinomycin D and did not persist 24 h later. Long-term activation of protein kinase C by 2 h exposure to PMA caused a dose-dependent increase in Na/H antiporter activity 24 h later. This latter effect was due to protein kinase C activation in that it was inhibited by sphingosine and was not seen with 4 alpha-PMA, an inactive analogue. The chronic effect of PMA was inhibited by 10 nM actinomycin D or 7 microM cycloheximide. Proximal tubule cells exposed to PMA for 2 h demonstrated a two- to threefold increase in Na/H antiporter mRNA (mRNANa/H) abundance 4 h later. In conclusion, short-term activation of protein kinase C leads to a transient increase in Na/H antiporter activity that is independent of transcription and translation, whereas long-term activation of protein kinase C causes a persistent increase in antiporter activity that is dependent on transcription and translation and is associated with increased mRNANa/H abundance. This latter effect may mediate increased […]

Long-Term Activation of Protein Kinase C Causes Chronic Na/H Antiporter Stimulation in Cultured Proximal Tubule Cells

Shigeo Horie, Orson Moe,* R. Tyler Miller, and Robert J. Alpern
*Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75235; and *Department of Veterans Affairs Medical Center, Dallas, Texas 75216*

Abstract

To examine the role of protein kinase C as a chronic regulator of proximal tubule Na/H antiporter activity, the effect of phorbol 12-myristate 13-acetate (PMA) on the Na/H antiporter was studied in cultured proximal tubule cells. Short-term activation of protein kinase C by 5 min exposure to PMA caused an acute increase in Na/H antiporter activity that was not prevented by cycloheximide or actinomycin D and did not persist 24 h later. Long-term activation of protein kinase C by 2 h exposure to PMA caused a dose-dependent increase in Na/H antiporter activity 24 h later. This latter effect was due to protein kinase C activation in that it was inhibited by sphingosine and was not seen with 4α-PMA, an inactive analogue. The chronic effect of PMA was inhibited by 10 nM actinomycin D or 7 μM cycloheximide. Proximal tubule cells exposed to PMA for 2 h demonstrated a two- to threefold increase in Na/H antiporter mRNA ($mRNA_{Na/H}$) abundance 4 h later. In conclusion, short-term activation of protein kinase C leads to a transient increase in Na/H antiporter activity that is independent of transcription and translation, whereas long-term activation of protein kinase C causes a persistent increase in antiporter activity that is dependent on transcription and translation and is associated with increased $mRNA_{Na/H}$ abundance. This latter effect may mediate increased Na/H antiporter activity in a number of chronic conditions. (*J. Clin. Invest.* 1992. 89:365–372.) Key words: memory • phorbol esters • cycloheximide • actinomycin D • Northern blot

Introduction

The proximal tubule is responsible for the majority of bicarbonate reabsorption in the kidney (1). A number of chronic conditions associated with increased proximal tubular H secretory capacity, including chronic metabolic and respiratory acidosis, chronic K deficiency, and chronic hyperfiltration, are all associated with adaptations in proximal tubular function (2–16). These adaptations include increased activities of the apical membrane Na/H antiporter and Na/citrate cotransporter, the basolateral membrane $Na/3HCO_3$ cotransporter, phosphate-dependent glutaminase, and phosphoenolpyruvate carboxykinase (2–14). A common feature of these proteins is that they all contribute to net acid excretion, either through acid secretion or ammonia synthesis. These adaptations persist after the transporter or enzyme has been removed from the inducing milieu. Kinetic studies on the transporters have found that substrate affinities are unaffected and V_{max} is increased (3, 6, 8, 9, 11).

We have recently developed a tissue culture model for studying these adaptations, in which rabbit proximal tubule cells in primary culture were incubated in control or acid media for 48 h (17). The results demonstrated an acid incubation–induced increase in Na/H antiporter activity that persisted after removal from the acid milieu and was dependent on protein synthesis. The adaptation was tissue specific in that it occurred in primary cultures of rabbit proximal tubule cells and in two renal cell lines but not in fibroblasts (17, 18). The adaptation was associated with a threefold increase in the abundance of mRNA encoding an Na/H antiporter ($mRNA_{Na/H}$)[1] (18).

The above studies together suggest that chronic regulation of proximal tubule acidification in a number of conditions is mediated by regulation of synthesis of a number of key transporters and enzymes. The cellular signaling mechanisms responsible for such adaptations have not been addressed. The association of these adaptations with proximal tubule cell hypertrophy suggests a possible role of protein kinase C (19). High levels of diacylglycerol, the endogenous activator of protein kinase C, have been found in the hyperfiltering kidney (12). For these reasons, the present studies examined the effect of chronic activation of protein kinase C on Na/H antiporter activity in proximal tubule cells in primary culture. The results demonstrate that, whereas short-term activation of protein kinase C causes a transient increase in Na/H antiporter activity that is independent of transcription and translation, more prolonged activation of protein kinase C causes a chronic persistent increase in Na/H antiporter activity that is dependent on transcription and translation and is associated with increased abundance of the $mRNA_{Na/H}$.

Methods

Cell culture. Primary cultures of renal proximal tubular cells were prepared as described previously (17). Briefly, 4–6-wk-old male New Zealand rabbits were killed by decapitation. Cortex was trimmed from kidneys, sliced with a Stadie-Riggs tissue slicer, and rinsed in ice-cold culture media. Cortical sections were incubated in culture media with 0.1% Type I collagenase for 40 min at 37°C with moderate shaking. The tissue suspension was centrifuged on an isosmotic 50% Percoll gradient (vol/vol) at 20,000 *g* for 30 min. Proximal tubule fragments were aspirated from the F4 fraction (20), washed, diluted in culture media to 3,000 tubules/ml, inoculated onto glass coverslips in 35-mm culture dishes and incubated at 37°C in a 95% air/5% CO_2 atmosphere.

Address reprint requests to Robert J. Alpern, M.D., Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8856. Dr. Horie's present address is Department of Urology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Received for publication 4 June 1991 and in revised form 25 September 1991.

1. *Abbreviations used in this paper:* BCECF, (2,7)-biscarboxyethyl-5(6)-carboxyfluorescein; $mRNA_{Na/H}$, Na/H antiporter mRNA.

Culture media consisted of a 1:1 (vol/vol) mixture of DME and Ham's F12 supplemented with 5 μg/ml insulin, 50 nM hydrocortisone, 35 μg/ml transferrin, 29 nM sodium selenite, 20 μM ethanolamine, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Fetal bovine serum was added to 3% (vol/vol) to the culture media for the first 3 d to allow better cell attachment. Medium was changed after 3 d and then on alternate days. After achieving confluence in 10–12 d, cells were rendered quiescent by removal of insulin and hydrocortisone for 2 d before and during the experimental protocol (21). Culture medium was changed on the day of experiment.

Measurement of cytoplasmic pH (pH_i). Continuous measurement of pH_i was accomplished using the pH-sensitive fluorescent dye, BCECF [(2,7)-biscarboxyethyl-5(6)-carboxyfluorescein] as described previously (17). Cells were loaded with the acetoxymethyl derivative of BCECF (10 μM) for 30 min at 37°C. The coverslip with cells then was placed in a plastic cuvette in a thermostatically controlled cuvette holder (37°C) in a computer-controlled spectrofluorimeter (model SLM 8000C; SLM Instruments Inc., Urbana, IL) at a 30° angle to the excitation beam. pH_i was estimated from the ratio of fluorescence with excitation at wavelengths of 500 nm and 450 nm, with emission at 530 nm. Slit widths were 4 nm. Background fluorescence was measured before dye loading and subtracted from fluorescence intensity at each excitation wavelength. Background fluorescence was < 0.1% of the total fluorescence at each wavelength. During pH_i measurement, cells were continuously perfused at 20 ml/min by solutions at 37°C. Rate of dye leakage from the cells was ~ 4%/min. There was no apparent time-dependent change in the fluorescent ratio over the time of the study. Calibration of the BCECF excitation ratio was determined using the K/nigericin technique as described (22).

Assay of Na/H antiporter activity. Na/H antiporter activity was assayed as the initial rate of Na-dependent pH_i increase after an acid load in the absence of CO_2/HCO_3. A typical tracing is depicted in Fig. 1. The composition of solutions used for the assay were as follows: (*a*) standard Na solution (mM): Na 137.7, K 4.5, Ca 1.1, Mg 1.54, Cl 147.5, Hepes 15; (*b*) Na-free solution: Na was replaced by choline; (*c*) Na-free NH_3/NH_4^+ solution: 20 mM NH_4^+ replaced 20 mM choline. All solutions were adjusted to pH 7.40 by titration with NMDG-OH at 37°C. Cells were first perfused with the standard Na solution to measure baseline pH_i, then with the Na-free NH_3/NH_4^+ solution for 5 min, and finally by the Na-free solution. On removal of NH_3/NH_4^+, pH_i decreased and reached a steady nadir. There was no pH_i recovery before Na addition in any study, implying either absent or very low activity of an H pump. Subsequent change to the standard Na solution caused rapid cell pH_i recovery, which was due to the Na/H antiporter. This was inhibited 85% by 1 mM amiloride (17) and 100% by 0.1 mM ethylisopropyl amiloride. The initial rate of this Na-dependent cell pH change (dpH_i/dt) was calculated by drawing a line tangent to the initial deflection (over 5 s).

The intracellular buffer capacity (β, mmol/l · pH) was determined from the pH_i response to NH_3/NH_4^+ removal in the above assay (23) using the formula: $\beta = [NH_4^+]_i/\Delta pH_i$, where $[NH_4^+]_i$ is the intracellular concentration of NH_4^+ just before NH_3/NH_4^+ removal, calculated as $[NH_4^+]_i = [NH_4^+]_o \cdot 10^{(7.4-pHi)}$; and ΔpH_i is the pH_i change on removal of NH_4^+. Na/H antiporter activity is the product of dpH_i/dt and buffer capacity. A change in dpH_i/dt is interpreted to reflect a difference in Na/H antiporter activity when cell buffer capacities in two conditions are similar.

Isolation and analysis of $mRNA_{Na/H}$. For these studies, cells were grown on 100-mm plastic culture plates. Proximal cell monolayers were lysed in guanidinium thiocyanate/*N*-lauroyl sarcosine/mercaptoethanol, and the resulting slurry was centrifuged through a 5.7 M cesium chloride cushion. The resulting RNA-enriched pellet was further purified by phenol-chloroform extraction and ethanol precipitation. PolyA$^+$RNA was selected by oligo-dT affinity chromatography. For RNA blot hybridization, polyA$^+$RNA from control and experimental cells was electrophoresed on formaldehyde-agarose gels and transferred to nylon filters (Genescreen; New England Nuclear, Boston, MA).

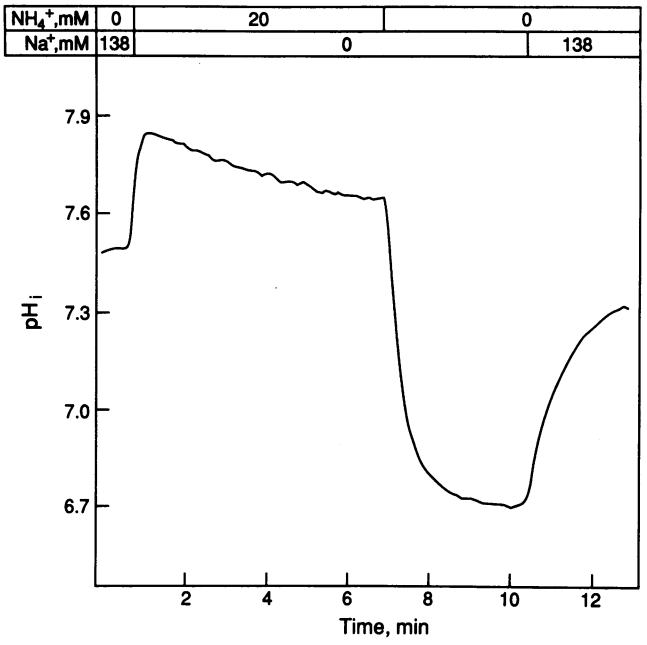

Figure 1. Assay of Na/H antiporter activity: typical tracing. pH_i (*y*-axis) is plotted as a function of time (*x*-axis). Cells are initially perfused with a Na-containing solution. At 1 min, Na is removed from the perfusate and 20 mM NH_4Cl is added, leading to a rapid cell alkalinization followed by a slow cell acidification. NH_4Cl then is removed from the perfusate, leading to a rapid cell acidification. When pH_i stabilizes, Na is added, leading to a rapid cell alkalinization that is mediated by the Na/H antiporter. All solutions are free of exogenous CO_2/HCO_3.

A single-stranded, uniformly ^{32}P-labeled DNA, synthesized as described previously (24), was used as a probe for RNA blotting. A 713-bp BamHI/SacI fragment of the C28 Na/H antiporter cDNA (25) has been subcloned into an M13 bacteriophage vector, allowing production of a single-stranded sense DNA template. A specific oligonucleotide was used to prime the synthesis of the antisense probe from the single-stranded DNA. A single-stranded, uniformly labeled β-actin probe was prepared in a similar fashion except that the synthesis was primed by an M13 universal primer. After prehybridization with 5× standard saline citrate (SSC; 0.75 M NaCl and 75 mM Na_3 citrate), 5× Denhardt's (1 mg/ml each of Ficoll, PVP, and BSA), and 0.5% SDS at 42°C for 4 h, filters were hybridized with 30–40 × 10^6 cpm of probe in the above solution at 42°C for 12–16 h. Nylon filters were then washed (1× SSC, 1% SDS for 15 min at 20°C; 0.1× SSC, 1% SDS for 1 h at 60°C) and subjected to autoradiography. The blots were sequentially probed for Na/H antiporter and β-actin. Densitometric analyses of autoradiograms were made on a laser densitometer with a software program (software, model GS-365-W; both densitometer and software from Hoefer Scientific Instruments, San Francisco, CA), and all Na/H antiporter signals were normalized for β-actin.

Statistics. Data are reported as mean±SE. Statistical significance was judged from unpaired Student's *t* test. One-way analysis of variance was performed when indicated.

Materials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted: collagenase (Type I) from Boehringer Mannheim Biochemicals, Indianapolis, IL; Percoll from Pharmacia LKB Biotechnology Inc., Piscataway, NJ; penicillin and streptomycin from Whittaker Bioproducts Inc., Walkersville, MD; culture media from Gibco Laboratories Life Technology Inc., Grand Island, NY; culture dishes from Corning Glass Works, Corning, NY; acetoxymethyl ester of BCECF (BCECF-AM) from Molecular Probes Inc., Eugene, OR; and phorbol 12-myristate 13-acetate (PMA) and

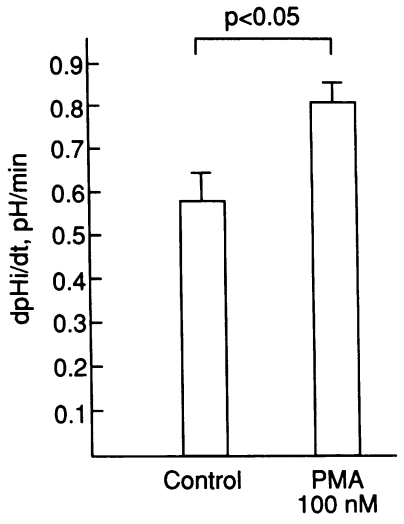

Figure 2. Effect of short-term activation of protein kinase C on Na/H antiporter activity. Results are expressed as the initial rate of change in pH_i (dpH_i/dt) (*y*-axis) in response to Na addition. Cells were incubated in either PMA (100 nM) or vehicle (0.1% DMSO) for 5 min after acid load.

4α-PMA from LC Services Corp., Woburn, MA. BCECF-AM, PMA, and 4α-PMA were dissolved in DMSO to make stock solutions. Sphingosine was used in a 1:1 molar complex with BSA as described elsewhere (26).

Results

Short-term activation of protein kinase C acutely increases Na/H antiporter activity. Initial studies examined the acute effect of short-term activation of protein kinase C on Na-dependent pH_i recovery from an acid load. For these studies protein kinase C was activated by addition of the phorbol ester, phorbol 12-myristate 13-acetate (PMA). After 5 min of perfusion with the Na-free NH_3/NH_4^+ solution, NH_3/NH_4^+ was removed and cells achieved a stable acid pH_i. Cells were then incubated with either vehicle or PMA in Na-free solution for 5 min, and dpH_i/dt was measured on Na addition. Fig. 2 summarizes the results. 5 min exposure to 100 nM PMA significantly increased dpH_i/dt from 0.58±0.06 to 0.80±0.05 pH unit/min ($P < 0.05$). Baseline pH_i (before NH_3/NH_4^+ addition), trough pH_i (just before Na addition), and buffer capacities were similar in the two groups (Table I).

To examine whether this effect of PMA was related to activation of protein kinase C or merely represented a nonspecific effect of phorbol esters, experiments were repeated with an analogue of PMA, 4α-PMA, which does not activate protein kinase C. In these experiments, dpH_i/dt was 0.51±0.03 in control cells and 0.43±0.05 in cells treated with 4α-PMA (NS, Table I). Thus, short-term activation of protein kinase C leads to an acute increase in Na/H antiporter activity in proximal tubule cells. This finding agrees with those of other studies demonstrating acute protein kinase C–induced antiporter activation in the renal proximal tubule (27, 28).

Given the rapidity of this effect, it is unlikely to be due to synthesis of gene products. To confirm this, we tested whether inhibition of transcription or translation prevented the PMA-induced increase in Na/H antiporter activity. In the first set of studies, cells were pretreated with 10 nM actinomycin D for 1 h before PMA addition to inhibit transcription. This concentration of actinomycin D inhibited the chronic response to PMA (see below). Higher concentrations of actinomycin D were toxic to the cells. Fig. 3 shows that in this setting PMA-induced stimulation of Na/H antiporter activity was still present. Baseline and trough pH_i and buffer capacities were similar in control and experimental cells (Table I).

In a second set of studies, control and PMA-treated cells were pretreated with 7 μM cycloheximide for 1 h before the experiment to inhibit translation. This concentration of cycloheximide inhibited most of protein synthesis in A6 cells and primary cultures of chick renal cells (29, 30). As shown in Fig. 3, cycloheximide did not prevent the PMA-induced increase in Na/H antiporter activity. Buffer capacities and baseline and trough pH_i were not different among the groups (Table I). Thus, short-term activation of protein kinase C causes an acute increase in Na/H antiporter activity that requires neither transcription nor translation and is most likely due to phosphorylation (31).

Table I. Acute Effects of PMA on Na/H Antiporter Activity

	n	Baseline pH_i	Trough pH_i	dpH_i/dt	β
				pH/min	*mmol/l·pH*
PMA × 5 min					
Control	5	7.47±0.03	6.66±0.03	0.58±0.06	18.3±1.5
PMA, 100 nM	5	7.39±0.03	6.64±0.06	0.80±0.05*	24.4±2.9
4α-PMA × 5 min					
Control	6	7.50±0.07	6.77±0.08	0.51±0.03	19.7±4.0
4α-PMA, 100 nM	6	7.51±0.06	6.79±0.05	0.43±0.05	17.4±1.6
PMA × 5 min: 10 nM actinomycin D					
Control	4	7.44±0.07	6.67±0.07	0.49±0.03	18.7±2.2
PMA, 100 nM	4	7.47±0.04	6.73±0.05	0.58±0.03*	16.6±1.7
PMA × 5 min: 7 μM cycloheximide					
Control	4	7.49±0.03	6.81±0.01	0.52±0.02	18.6±2.7
PMA, 100 nM	3	7.44±0.03	6.75±0.02	0.71±0.02‡	21.0±3.2

Values are mean±SE.
* $P < 0.05$ vs. control; ‡ $P < 0.001$ vs. control.
PMA, phorbol 12-myristate 13-acetate.

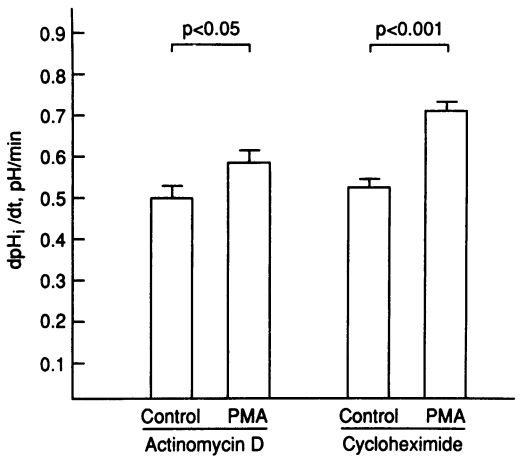

Figure 3. Effect of actinomycin D or cycloheximide on acute PMA-induced increase in Na/H antiporter activity. Actinomycin D (10 nM) or cycloheximide (7 μM) was added 1 h before the assay. Data are presented as in Fig. 2.

Long-term activation of protein kinase C causes a chronic persistent increase in Na/H antiporter activity. To examine whether protein kinase C activation causes a persistent stimulation of Na/H antiporter activity, we examined Na/H antiporter activity 24 h after removal of PMA. Cells were preincubated with PMA or vehicle added to the culture media. After a set time, PMA or vehicle was removed and cells were washed twice and refed with fresh culture media. Na/H antiporter activity was then assayed 24 h later.

In the first studies, 100 nM PMA was added for 5 min. 24 h later, cells were loaded with BCECF and studied. Baseline and trough pH_i were not different between control and PMA-pretreated cells (Table II). Na/H antiporter activities were also not different (Fig. 4). Once again, buffer capacities were similar in the two groups (Table II). Thus, 5 min exposure to PMA caused an acute activation of the Na/H antiporter, which was transient in that it was no longer present 24 h later.

To examine whether a more chronic activation of protein kinase C would lead to a more persistent activation of the Na/H antiporter, cells were exposed to PMA for 2 h. Once again, after 2 h exposure, cells were washed twice and refed. Antiporter activity was assessed 24 h later. Fig. 5 shows the results. Compared with a control rate of 0.54±0.03 pH unit/min, 2 h preincubation with 30 nM PMA caused a 22% stimulation, and 2 h preincubation with 100 nM PMA caused a 44% increase in Na/H antiporter activity. Baseline and trough cell pH and buffer capacities were not different between the groups (Table II).

PMA-induced persistent increase in Na/H antiporter activity is through activation of protein kinase C. To address whether the above effect of 2 h PMA preincubation was due to activation of protein kinase C, two sets of studies were performed. First, we examined the effect of a 2-h application of 4α-PMA, the inactive analogue. 2-h incubation with 4α-PMA failed to increase Na/H antiporter activity (Table II).

In a second set of studies, we examined the effect of inhibition of protein kinase C with sphingosine (32) on the ability of 2-h treatment with PMA to chronically increase Na/H antiporter activity. Cells were treated with 10 μM sphingosine with 10 μM albumin for 12 h before and then during the exposure to

Table II. Chronic Effects of PMA on Na/H Antiporter Activity

	n	Baseline pH_i	Trough pH_i	dpH_i/dt	β
				pH/min	*mmol/l·pH*
PMA × 5 min					
Control	8	7.50±0.02	6.68±0.02	0.67±0.03	12.9±0.9
PMA, 100 nM	8	7.45±0.03	6.68±0.03	0.73±0.06	12.4±0.7
PMA × 2 h					
Control	17	7.49±0.02	6.69±0.02	0.54±0.03	14.1±0.5
PMA, 30 nM	6	7.42±0.02	6.63±0.01	0.66±0.05*	14.0±0.6
PMA, 100 nM	17	7.43±0.02	6.65±0.02	0.78±0.04‡	12.5±0.6
4α-PMA × 2 h					
Control	4	7.53±0.06	6.78±0.05	0.52±0.06	15.5±2.4
4α-PMA, 100 nM	4	7.53±0.06	6.77±0.05	0.43±0.05	17.3±2.0
PMA × 2 h: 10 μM sphingosine					
Control, albumin	8	7.50±0.05	6.63±0.04	0.49±0.04	11.7±1.5
PMA, 100 nM, albumin	8	7.55±0.04	6.68±0.06	0.66±0.05*	10.6±0.7
Control, sphingosine	10	7.53±0.02	6.67±0.05	0.54±0.05	12.3±0.5
PMA, 100 nM, sphingosine	12	7.50±0.02	6.66±0.03	0.55±0.05	11.6±0.6
PMA × 2 h: 10 nM actinomycin D					
Control	8	7.42±0.03	6.68±0.01	0.56±0.04	16.0±0.8
PMA, 100 nM	8	7.43±0.02	6.70±0.02	0.59±0.04	15.3±1.0
PMA × 2 h: 7 μM cycloheximide					
Control	5	7.53±0.05	6.89±0.04	0.57±0.04	12.7±0.5
PMA, 100 nM	5	7.51±0.04	6.96±0.04	0.48±0.04	12.6±0.3

Values are mean±SE.
* $P < 0.05$ vs. control; ‡ $P < 0.001$ vs. control.

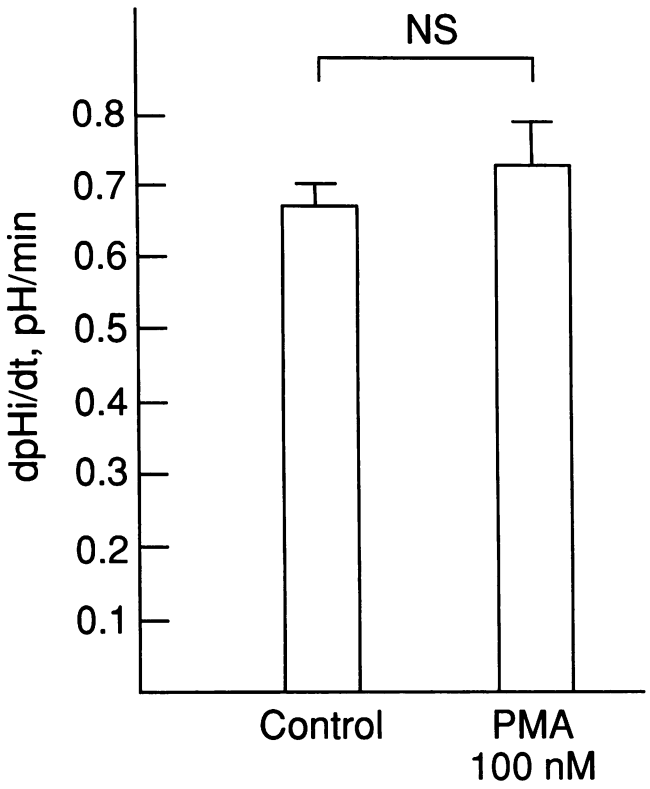

Figure 4. Chronic effect of short-term protein kinase C activation on Na/H antiporter activity. Na/H antiporter was assayed 24 h after 5 min exposure to 100 nM PMA or vehicle. Data are presented as in Fig. 2.

PMA (26). Control studies were performed with albumin alone. Fig. 6 shows that sphingosine prevented the PMA-induced increase in Na/H antiporter activity, whereas albumin had no effect. Once again, baseline and trough cell pH values and buffer capacities were identical in all groups (Table II). Thus, the chronic effect of PMA preincubation on Na/H antiporter activity is mediated by activation of protein kinase C.

Protein kinase C–induced chronic effect is dependent on transcription and translation. The observation that a 2-h treatment with PMA led to increased Na/H antiporter activity 24 h after the treatment raised the question of whether transcription and translation were involved in this process. To examine this question, two sets of studies were performed. First, actinomycin D was used to block transcription. Control and experimental cells were incubated with 10 nM actinomycin D 1 h before and during addition of PMA or vehicle. As can be seen in Fig. 7 and Table II, actinomycin D completely inhibited the PMA-induced stimulation of the Na/H antiporter.

In a second set of studies, 7 μM cycloheximide was added 1 h before and during preincubation in PMA or vehicle and then throughout the next 24 h to control and experimental cells. As shown in Fig. 7 and Table II, cycloheximide completely inhibited PMA-induced stimulation of Na/H antiporter activity. Thus, the effect of chronic protein kinase C activation to cause a chronic persistent increase in Na/H antiporter activity is dependent on synthesis of gene products.

Prolonged activation of protein kinase C leads to increased abundance of $mRNA_{Na/H}$. Sardet et al. (25) have cloned a cDNA for an Na/H antiporter. Because our effect of PMA on the Na/H antiporter was dependent on transcription and translation, we next examined whether there was an increase in abundance of this mRNA. Quiescent cells were incubated with either 100 nM PMA or vehicle for 2 h, washed twice, and then refed with culture media. 4 h after incubation, cells were harvested and Northern blot analysis was performed as described

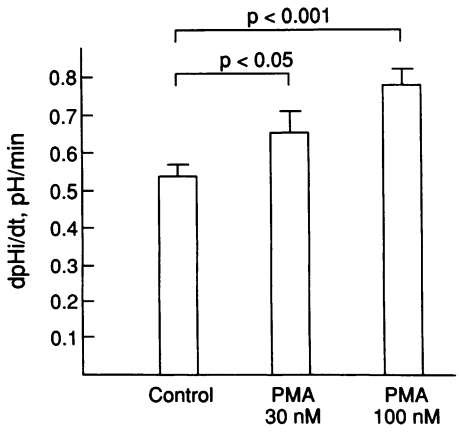

Figure 5. Chronic effect of long-term protein kinase C activation on Na/H antiporter activity. Na/H antiporter was assayed 24 h after 2 h exposure to 0, 30, or 100 nM PMA. Data are presented as in Fig. 2.

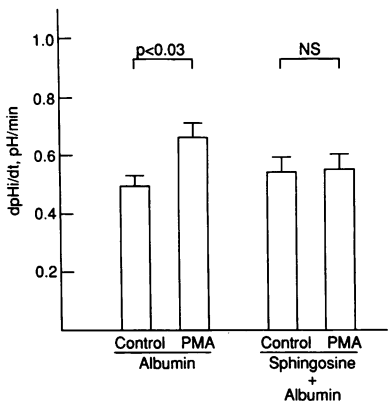

Figure 6. Effect of sphinogosine on PMA-induced chronic increase in Na/H antiporter activity. See Fig. 5 for experimental protocol. 10 μM sphingosine + 10 μM albumin or just 10 μM albumin were added 12 h before PMA (100 nM) and remained throughout PMA addition. Data are presented as in Fig. 2.

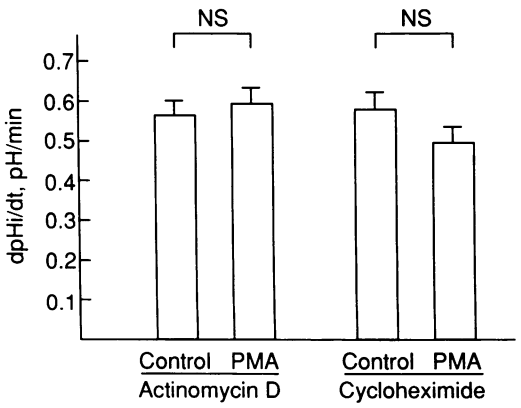

Figure 7. Effect of actinomycin D and cycloheximide on PMA-induced chronic stimulation of Na/H antiporter activity. See Fig. 5 for experimental protocol. Actinomycin D (10 nM) was added 1 h before and during PMA addition. Cycloheximide (7 μM) was added 1 h before and during PMA addition, and then during the following 24-h incubation. Data are presented as in Fig. 2.

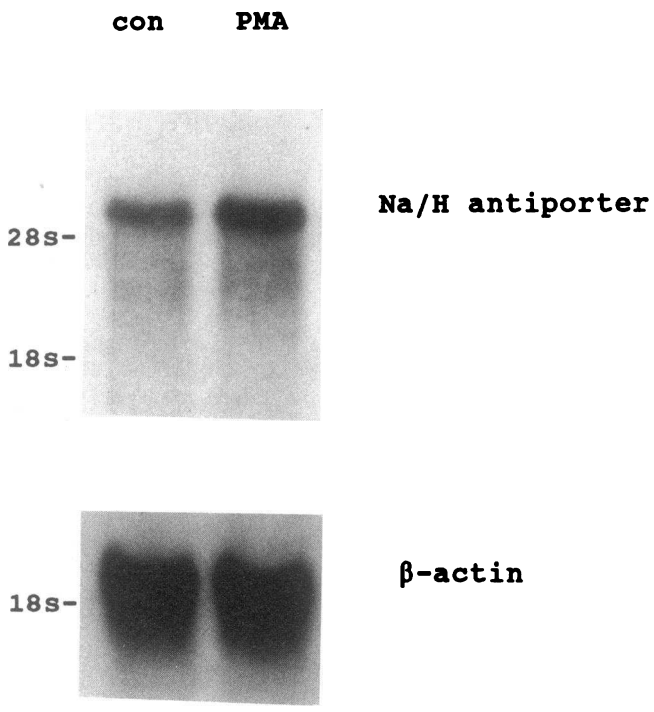

Figure 8. Effect of long-term activation of protein kinase C on Na/H antiporter mRNA abundance. Quiescent primary culture cells were treated for 2 h with either 100 nM PMA or vehicle. mRNA was prepared 4 h later and analyzed as described under Methods.

in Methods. Fig. 8 shows results of a typical experiment. When normalized for β-actin, $mRNA_{Na/H}$ abundance was increased 2.3-fold. Similar results were achieved in two other separate experiments (3.1- and 2.5-fold increases).

Discussion

Short-term activation of protein kinase C leads to acute activation of the Na/H antiporter. The present studies find that acute activation of protein kinase C by addition of phorbol esters leads to an immediate increase in Na/H antiporter activity in cultured proximal tubule cells. These results are similar to those found previously in proximal tubule suspensions and renal cortical apical membrane vesicles (27, 28) and in numerous other cell types (33). In addition, acute activation of protein kinase C (< 10 min) has been found to increase the rate of proximal tubule HCO_3 absorption (34, 35).

The rapidity of the acute effect suggests that it is mediated by phosphorylation of the Na/H antiporter and is independent of transcription or translation. Our results confirm this thesis by showing that actinomycin D and cycloheximide have no effect on the acute stimulation of Na/H antiporter activity. Sardet et al. (31) have shown in fibroblasts that activation of protein kinase C by phorbol esters leads to phosphorylation of the Na/H antiporter on serine residues. The renal Na/H antiporter has also been shown to be regulated by a number of other kinases, including cAMP-dependent protein kinase and calcium calmodulin–dependent multiprotein kinase (reviewed in reference 36).

Long-term activation of protein kinase C leads to a chronic persistent increase in Na/H antiporter activity. In addition to acute regulation, Na/H antiporter activity is regulated by a number of chronic conditions. Chronic metabolic acidosis, chronic respiratory acidosis, chronic K deficiency, and chronic increases in glomerular filtration rate are all associated with an adaptive increase in Na/H antiporter activity that is kinetically expressed as an increase in the V_{max} of the transporter (2–12). In addition, this altered antiporter activity persists after the transporter is removed from the experimental environment.

This latter observation suggests that these adaptations represent a form of long-term renal memory and may be similar to the mechanisms of long-term memory in the central nervous system. The molecular basis of memory has been extensively studied in Aplysia sensory neurons, where it is mediated by serotonin-induced changes in cAMP that, secondarily, regulate K channel activity. Short-term application (5 min) of serotonin or cAMP analogues leads to an acute decrease in K channel activity that is transient and independent of transcription and translation, whereas addition of serotonin or cAMP analogues for 2 h leads to a more prolonged change in K channel activity that is dependent on transcription and translation (37–39). In the present studies we adapted a similar protocol and found similar results. Activation of protein kinase C with a 5-min application of PMA caused a transient increase in Na/H antiporter activity that was independent of transcription and translation, whereas a 2-h application caused an increase in Na/H antiporter activity that persisted 24 h later and was dependent on transcription and translation. Thus, in both these forms of neural and renal memory, one protein kinase is able to regulate transporter activity acutely by phosphorylation and chronically by regulation of gene expression. Long-term potentiation of synaptic transmission in the hippocampus is also mediated by chronic activation of protein kinase C (40).

The inhibition of the chronic stimulation in Na/H antiporter activity by cycloheximide and actinomycin D suggests that transcription and translation are required. However, the synthesized protein may not be the Na/H antiporter but, rather, could be a regulatory protein. In Aplysia sensory neurons, long-term regulation of K-channel activity requires protein synthesis, but the synthesized protein appears to be a regulatory protein rather than the K channel per se (41). To examine this issue in the present studies, we examined the effect of 2 h PMA on the abundance of the mRNA corresponding to the Na/H antiporter gene of Sardet et al. (25). It is presently unresolved whether this cDNA encodes the apical membrane Na/H antiporter of proximal tubule cells, and recent evidence suggests that it may not. Epithelial apical membrane Na/H antiporters are amiloride resistant, whereas the "housekeeping" Na/H antiporters present in nonpolar cells and epithelial basolateral membranes are amiloride sensitive (42). The Na/H antiporter of Sardet et al. (25) is present in fibroblasts and is expressed as an amiloride-sensitive Na/H antiporter (43). In addition, a second Na/H antiporter gene has recently been cloned from a rabbit intestinal cDNA library (43). Thus, it seems likely that the gene cloned by Sardet et al. encodes the housekeeping Na/H antiporter and that there may be a separate gene for the renal apical membrane Na/H antiporter, possibly similar to the rabbit intestinal antiporter.

In proximal tubule and renal cell lines, acid incubation increases the activities of both amiloride-sensitive and -resistant Na/H antiporters (18, 44). Acid incubation of renal cells also causes an increase in abundance of the mRNA corresponding to Sardet's clone (18). In the present studies, application of PMA for 2 h caused a two- to threefold increase in Na/H anti-

porter mRNA abundance 4 h later. Thus, at least one Na/H antiporter mRNA is regulated by PMA. This response is similar to that seen with chronic acid incubation. Further studies are required to examine whether mRNA corresponding to the other Na/H antiporter genes is also regulated and define the roles of Na/H antiporter protein synthesis versus synthesis of regulatory proteins. The current results do not address the question of whether the observed increase in mRNA abundance is due to increased rates of transcription or increased message stability.

Protein kinase C has been demonstrated to regulate expression of a number of different genes. A number of consensus sequences present in the 5′ flanking regions of these genes have been found to mediate this regulation of gene expression (45–47). Most commonly, protein kinase C activation of genes is mediated by binding of AP-1, or fos/jun dimers, to an AP-1 binding site in the regulatory region of the gene (45). We have recently found that the promoter/enhancer region of the gene for the human Na/H antiporter contains three consensus sequences for AP-1 binding sites (48).

Chronic activation of protein kinase C with high concentrations of phorbol esters leads to downregulation of protein kinase C (49). This raises the question of whether effects of chronic protein kinase C activation are physiologically relevant. However, it is likely that physiological activation of protein kinase C is not of sufficient magnitude to eliminate protein kinase C activity. Our observed effect of chronic PMA application is due to protein kinase C activation rather than downregulation, as it is blocked by sphingosine. If protein kinase C downregulation were responsible for the chronic increase in Na/H antiporter activity, one would expect that sphingosine inhibition would mimic the effect.

Baum and Hays (50) and Wang and Chan (35) found that application of PMA for > 15 and > 30 min, respectively, caused inhibition of HCO_3 absorption in the perfused proximal tubule. It is difficult to reconcile their results with ours, but the explanation may reside in differences in the experimental protocol (timing of PMA addition and removal). In addition, it is possible that perfused tubules do not synthesize proteins normally. Lastly, since these authors measured HCO_3 absorption rather than Na/H antiporter activity, it is possible that their late effect was due to inhibition of another transporter (NaK-ATPase, Na/3HCO_3 cotransporter, H-ATPase).

Similar to the effect of chronic protein kinase C activation, chronic incubation (24–48 h) of proximal tubule cells in acid media leads to an increase in Na/H antiporter activity that is dependent on transcription and translation and is associated with increased abundance of mRNA for the Na/H antiporter (17, 18, 44). In preliminary results, we have found that this increase in antiporter activity is blocked by inhibition of protein kinase C (51). Together with the present results, this suggests that protein kinase C activation may play a major role in the adaptation to chronic acidosis. In addition, high renal cortical diacylglycerol levels have been found in hyperfiltration, a condition also associated with chronic increases in Na/H antiporter activity (12).

In summary, the present studies show that short-term activation of protein kinase C leads to acute transient activation of the Na/H antiporter that is independent of transcription and translation, whereas more long-term activation of protein kinase C leads to a chronic persistent increase in Na/H antiporter activity that is dependent on transcription and translation. This latter chronic effect may play a role in the increase in Na/H antiporter activity seen in chronic acidosis, chronic K deficiency, and chronic hyperfiltration.

Acknowledgments

We appreciate the technical assistance of Martha Ferguson and Ladonna Crowder.

This work was supported by grants DK-39298 and DK-41726 from the National Institutes of Health. Shigeo Horie was supported by a National Kidney Foundation fellowship. Orson Moe was supported by a Canadian Kidney Foundation fellowship.

References

1. Alpern, R. J. 1990. Cell mechanisms of proximal tubule acidification. *Physiol. Rev.* 70:79–114.

2. Cohn, D. E., S. Klahr, and M. R. Hammerman. 1983. Metabolic acidosis and parathyroidectomy increase Na^+/H^+ exchange in brush border vesicles. *Am. J. Physiol.* 245(*Renal Fluid Electrolyte Physiol. 14*):F217–F222.

3. Tsai, C. J., H. E. Ives, R. J. Alpern, V. J. Yee, D. G. Warnock, and F. C. Rector, Jr. 1984. Increased V_{max} for Na^+/H^+ antiporter activity in proximal tubule brush border vesicles from rabbits with metabolic acidosis. *Am. J. Physiol.* 147(*Renal Fluid Electrolyte Physiol. 16*):F339–F343.

4. Kinsella, J. L., T. Cujkid, and B. Sactor. 1984. Na^+-H^+ exchange activity in renal brush border membrane vesicles in response to metabolic acidosis: the role of glucocorticoids. *Proc. Natl. Acad. Sci. USA.* 81:630–634.

5. Preisig, P. A., and R. J. Alpern. 1988. Chronic metabolic acidosis causes an adaptation in the apical membrane Na/H antiporter and basolateral membrane $Na(HCO_3)_3$ symporter in the rat proximal convoluted tubule. *J. Clin. Invest.* 82:1445–1453.

6. Akiba, T., V. K. Rocco, and D. G. Warnock. 1987. Parallel adaptation of the rabbit renal cortical sodium/proton antiporter and sodium/bicarbonate cotransporter in metabolic acidosis and alkalosis. *J. Clin. Invest.* 80:308–315.

7. Krapf, R. 1989. Mechanisms of adaptation to chronic respiratory acidosis in the rabbit proximal tubule. *J. Clin. Invest.* 83:890–896.

8. Ruiz, O. S., J. A. L. Arruda, and Z. Talor. 1989. Na-HCO_3 cotransport and Na-H antiporter in chronic respiratory acidosis and alkalosis. *Am. J. Physiol.* 256(*Renal Fluid Electrolyte Physiol. 25*):F414–F420.

9. Soleimani, M., J. A. Bergman, M. A. Hosford, and T. D. McKinney. 1990. Potassium depletion increases luminal Na^+/H^+ exchange and basolateral Na^+:CO_3:HCO_3^- cotransport in rat renal cortex. *J. Clin. Invest.* 86:1076–1083.

10. Preisig, P. A., and R. J. Alpern. 1991. Increased Na/H antiporter and Na/3HCO_3 symporter activities in chronic hyperfiltration: a model of cell hypertrophy. *J. Gen. Physiol.* 97:195–217.

11. Harris, R. C., J. L. Seifter, and B. M. Brenner. 1984. Adaptation of Na^+-H^+ exchange in renal microvillus membrane vesicles. *J. Clin. Invest.* 74:1979–1987.

12. Salihagic, A., M. Mackovic, H. Banfic, and I. Sabolic. 1988. Short-term and long-term stimulation of Na^+-H^+ exchange in cortical brush-border membranes during compensatory growth of the rat kidney. *Pfluegers Arch. Eur. J. Physiol.* 413:190–196.

13. Jenkins, A. D., T. P. Dousa, and L. H. Smith. 1985. Transport of citrate across renal brush border membrane: effects of dietary acid and alkali loading. *Am. J. Physiol.* 249(*Renal Fluid Electrolyte Physiol. 18*):F590–F595.

14. Alpern, R. J., D. G. Warnock, and F. C. Rector, Jr. 1986. Renal acidification mechanisms. *In* The Kidney. 3rd ed. B. M. Brenner and F. C. Rector, Jr., editors. W.B. Saunders, Philadelphia, PA. 206–249.

15. Cogan, M. G. 1984. Chronic hypercapnia stimulates proximal bicarbonate reabsorption in the rat. *J. Clin. Invest.* 74:1942–1947.

16. Chan, Y. L., B. Biagi, and G. Giebisch. 1982. Control mechanisms of bicarbonate transport across the rat proximal convoluted tubule. *Am. J. Physiol.* 242(*Renal Fluid Electrolyte Physiol. 11*):F532–F543.

17. Horie, S., O. Moe, A. Tejedor, and R. J. Alpern. 1990. Preincubation in acid medium increases Na/H antiporter activity in cultured renal proximal tubule cells. *Proc. Natl. Acad. Sci. USA.* 87:4742–4745.

18. Moe, O. W., R. T. Miller, S. Horie, A. Cano, P. A. Preisig, and R. J. Alpern. 1991. Differential regulation of the Na/H antiporter by acid in renal epithelial cells and fibroblasts. *J. Clin. Invest.* 88:1703–1708.

19. Fine, L. 1986. The biology of renal hypertrophy. *Kidney Int.* 29:619–634.

20. Vinay, P., A. Gougoux, and G. Lemieux. 1981. Isolation of a pure suspension of rat proximal tubules. *Am. J. Physiol.* 241(*Renal Fluid Electrolyte Physiol. 10*):F403–F411.

21. Fine, L. G., B. Badie-Desfooly, A. G. Lowe, A. Hamzeh, J. Wells, and S. Salehmoghaddam. 1985. Stimulation of Na^+/H^+ antiport is an early event in

hypertrophy of renal proximal tubular cells. *Proc. Natl. Acad. Sci. USA.* 82:1736–1740.

22. Alpern, R. J. 1985. Mechanism of basolateral membrane $H^+/OH^-/HCO_3^-$ transport in the rat proximal convoluted tubule. *J. Gen. Physiol.* 86:613–636.

23. Roos, A., and W. F. Boron. 1981. Intracellular pH. *Physiol. Rev.* 61:296–434.

24. Hobbs, H. H., M. S. Brown, J. L. Goldstein, and D. W. Russell. 1986. Deletion of exon encoding cystein-rich repeat of low density lipoprotein receptor alters its binding specificity in a subject with familial hypercholesterolemia. *J. Biol. Chem.* 261:13114–13120.

25. Sardet, C., A. Franchi, and J. Pouyssegur. 1989. Molecular cloning, primary structure, and expression of the human growth factor–activatable Na^+/H^+ antiporter. *Cell.* 56:271–280.

26. Lowe, J. H. N., C. L. Huang, and H. E. Ives. 1990. Sphingosine differentially inhibits activation of the Na^+/H^+ exchanger by phorbol esters and growth factors. *J. Biol. Chem.* 265:7188–7194.

27. Mellas, J., and M. R. Hammerman. 1986. Phorbol ester–induced alkalinization of canine renal proximal tubular cells. *Am. J. Physiol.* 250(*Renal Fluid Electrolyte Physiol. 19*):F451–F459.

28. Weinman, E. J., and S. Shenolikar. 1986. Protein kinase C activates the renal apical membrane Na^+/H^+ exchanger. *J. Membr. Biol.* 93:133–139.

29. Verrey, F., E. Schaerer, P. Zoerkler, M. P. Paccolat, K. Geering, J. P. Kraehenbuhl, and B. C. Rossier. 1987. Regulation by aldosterone of Na^+, K^+-ATPase mRNAs, protein synthesis, and sodium transport in cultured kidney cells. *J. Cell Biol.* 104:1231–1237.

30. Noronha-Blob, L., and B. Sacktor. 1986. Inhibition by glucocorticoids of phosphate transport in primary cultured renal cells. *J. Biol. Chem.* 261:2164–2169.

31. Sardet, C., L. Lounillon, A. Franchi, and J. Pouyssegur. 1990. Growth factors induce phosphorylation of the Na^+/H^+ antiporter, a glycoprotein of 110 kD. *Science (Wash. DC).* 247:723–726.

32. Hannun, Y. A., C. S. Greenberg, and R. M. Bell. 1987. Sphingosine inhibition of agonist-dependent secretion and activation of human platelets implies that protein kinase C is a necessary and common event of the signal transduction pathways. *J. Biol. Chem.* 262:13620–13626.

33. Grinstein, S., and A. Rothstein. 1986. Mechanisms of regulation of the Na^+/H^+ exchanger. *J. Membr. Biol.* 90:1–12.

34. Liu, F. Y., and M. G. Cogan. 1990. Role of protein kinase C in proximal bicarbonate absorption and angiotensin signaling. *Am. J. Physiol.* 258(*Renal Fluid Electrolyte Physiol. 27*):F927–F933.

35. Wang, T., and Y. L. Chan. 1990. Time- and dose-dependent effects of protein kinase C on proximal bicarbonate transport. *J. Membr. Biol.* 117:131–139.

36. Weinman, E. J., W. Dubinsky, and S. Shenolikar. 1989. Regulation of the renal Na^+-H^+ exchanger by protein phosphorylation. *Kidney Int.* 36:519–525.

37. Montarolo, G., P. Goelet, V. F. Castellucci, J. Morgan, R. Kandel, and S. Schacher. 1986. A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia. Science (Wash. DC).* 234:1249–1254.

38. Schacher, S., V. F. Castellucci, and E. R. Kandel. 1988. cAMP evokes long-term facilitation in *Aplysia* sensory neurons that requires new protein synthesis. *Science (Wash. DC).* 240:1667–1669.

39. Goelet, P., V. F. Castellucci, S. Schacher, and E. R. Kandel. 1986. The long and the short of long-term memory—a molecular framework. *Nature (Lond.).* 322:419–422.

40. Malinow, R., D. V. Madison, and R. W. Tsien. 1988. Persistent protein kinase activity underlying long-term potentiation. *Nature (Lond.).* 335:820–824.

41. Sweatt, J. D., and E. R. Kandel. 1989. Persistent and transcriptionally-dependent increase in protein phosphorylation in long-term facilitation of *Aplysia* sensory neurons. *Nature (Lond.).* 339:51–54.

42. Haggerty, J. G., N. Agarwal, R. F. Reilly, E. A. Adelbert, and C. W. Slayman. 1988. Pharmacologically different Na/H antiporters on the apical and basolateral surfaces of cultured porcine kidney cells (LLC-PK_1). *Proc. Natl. Acad. Sci. USA.* 85:6797–6801.

43. Sardet, C., S. Wakayabashi, P. Fafournoux, L. Counillon, G. Pages, and J. Pouyssegur. 1991. Molecular properties of Na^+/H^+ aexchangers. *In* Acid-Base Balance. N. G. De Santo and G. Capasso, editors. Editoriale Bios, Cosenza, Italy. 13–20.

44. Igarashi, P., M. Ganz, and R. F. Reilly. 1990. Metabolic acidosis stimulates transport activity and transcript levels of the basolateral Na/H exchanger in LLC-PK_1 cells *J. Am. Soc. Nephrol.* 1:721. (Abstr.)

45. Curran, T., and B. R. Franza, Jr. 1988. Fos and jun: the AP-1 connection. *Cell.* 55:395–397.

46. Comb, M., N. C. Birnberg, A. Seasholtz, E. Herbert, and H. M. Goodman. 1986. A cyclic AMP- and phorbol ester–inducible DNA element. *Nature (Lond.).* 323:353–356.

47. Chiu, R., M. Imagawa, R. J. Imbra, J. R. Bockoven, and M. Karin. 1987. Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol esters. *Nature (Lond.).* 329:648–651.

48. Miller, R. T., L. Counillon, G. Pages, R. P. Lifton, C. Sardet, and J. Pouyssegur. 1991. Structure of the 5′-flanking regulatory region and gene for the human growth factor–activatable Na/H exchanger NHE-1. *J. Biol. Chem.* 266:10813–10819.

49. Huang, F. L., Y. Yoshida, J. R. Cunha-Melo, M. A. Beaven, and K. P. Huang. 1989. Differential down-regulation of protein kinase C isozymes. *J. Biol. Chem.* 264:4238–4243.

50. Baum, M., and S. R. Hays. 1988. Phorbol myristate acetate and dioctanoylglycerol inhibit transport in rabbit proximal convoluted tubule. *Am. J. Physiol.* 254(*Renal Fluid Electrolyte Physiol. 23*):F9–F14.

51. Moe, O., S. Horie, R. T. Miller, and R. J. Alpern. 1990. Characterization of the effect of chronic acid preincubation on Na/H antiporter activity. *J. Am. Soc. Nephrol.* 1:655. (Abstr.)