Na+/H+ exchanger (NHE) activity is regulated by several types of receptors directly coupled to distinct classes (i.e. Gα, Gβ, and Gγ) of heterotrimeric (αβγ) GTP-binding proteins (G proteins), which, upon activation, modulate production of various second messengers (e.g. cAMP, cGMP, diacylglycerol, inositol triphosphate, and Ca2+). Recently, four isoforms of the rat Na+/H+ exchanger were identified by molecular cloning. To examine their intrinsic responsiveness to G protein and second messenger stimulation, three of these isoforms, NHE-1, -2, and -3, were stably expressed in mutant Chinese hamster ovary cells devoid of endogenous NHE activity (AP-1 cells). Incubation of cells with either AIFα, a general agonist of G proteins, or cholera toxin, a selective activator of Gαq that stimulates adenylate cyclase, accelerated the rates of amiloride-inhibitable 22Na+ influx mediated by NHE-1 and -2, whereas they inhibited that by NHE-3. Similarly, short term treatment with phorbol 12-myristate 13-acetate, which mimics diacylglycerol activation of protein kinase C (PKC), or with agents (i.e. forskolin, 8-(4-chlorophenylthio)-cAMP, and isobutylmethylxanthine) that lead to activation of cAMP-dependent protein kinase (PKA) also stimulated transport by NHE-1 and NHE-2 but depressed that by NHE-3. The effects of phorbol 12-myristate 13-acetate were blocked by depleting cells of PKC or by inhibiting PKC using chelerythrine chloride, confirming a role for PKC in modulating NHE isoform activities. Likewise, the PKA antagonist, H-89, attenuated the effects of elevated cAMP, on NHE-1, -2, and -3, further demonstrating the regulation by PKA. Unlike cAMP, elevation of cGMP, by treatment with dibutyryl-cGMP or 8-bromo-cGMP had no influence on NHE isoform activities, thereby excluding the possibility of a role for cGMP-dependent protein kinase in these cells. These data support the concept that the NHE isoforms are differentially responsive to agonists of the PKA and PKC pathways.

Na+/H+ exchanger (NHE)3 activity is present in the plasma membrane of all mammalian cells and, depending on the cell type and membrane localization, fulfills several distinct physiological functions, including control of intracellular pH (pH i), maintenance of cellular volume, facilitation of cell proliferation in response to growth factor stimulation, and transepithelial Na+ reabsorption (reviewed in Ref. 1). This functional diversity is accomplished by the actions of distinct isoforms of the Na+/H+ exchanger.

To date, four members (NHE-1 to NHE-4) of this multigene family have been identified and characterized by cDNA cloning (2-5) and functional expression studies (6-9). More recently, the existence of a putative fifth (10) and possibly sixth (11) isoform have been revealed by chromosomal mapping in humans. Overall, they share ~40-60% amino acid identity (molecular mass ranging from ~81 to 93 kDa) and exhibit similar plasma membrane topologies, with 10-12 predicted N-terminal transmembrane-spanning regions and a large C-terminal cytoplasmic region. This latter region exhibits the greatest divergence in amino acid sequence among the isoforms and contains one or more potential sites for phosphorylation by different serine/threonine protein kinases.

Previous studies have revealed a wide variety of molecular signals, including neurotransmitters, growth factors, peptide hormones, chemoattractants, lectins, and osmotic shrinkage, that rapidly modulate Na+/H+ exchanger activity (for reviews, see Refs. 1 and 12). Many of these stimuli transmit their signals via interactions with plasma membrane receptors that are coupled to a diverse family of heterotrimeric (αβγ) GTP-binding proteins (G proteins) (for reviews, see Refs. 13 and 14). Receptor-mediated activation of G proteins leads to dissociation of α-GTP from the βγ subunits (which remain tightly associated) and their release from the receptor. These subunits (α or βγ), in turn, can directly bind and regulate a variety of effector molecules, such as Ca2+ and K+ channels, adenylate cyclase, cGMP phosphodiesterase, and phospholipase Cβ, thereby modulating intracellular ion levels and signaling pathways (i.e. cAMP, cGMP, diacylglycerol, inositol triphosphate, and Ca2+).

The response of the Na+/H+ exchanger following activation of different serinethreonine kinases is complex and dependent on cell type (reviewed in Refs. 5 and 12). In most nonepithelial cells, growth factors and phorbol esters that mediate their

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The abbreviations used are: NHE, Na+/H+ exchanger; BCECF, 2,7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; AM, acetoxymethyl-ester; cAMP, 3′(5′)-adenosine 3′-monophosphate; CTX, cholera toxin; PKA, cAMP-dependent protein kinase or protein kinase A; PKC, Ca2+/phospholipid-dependent protein kinase or protein kinase C; EIPA, 5-(N-ethyl-N-isopropyl) amiloride; pH i, intracellular pH; pH e, extracellular pH; α-MEM, α-minimal essential medium.
effects through PKC generally accelerate exchanger activity (15). Moreover, in some renal and intestinal epithelial cells, the apical exchanger is inhibited (16, 17) under conditions where the basolateral exchanger remains unaffected (18). However, agents that elevate intracellular cAMP (cAMP pathways) also provided contradictory results, with exchanger activity being stimulated (26–29) or depressed (29, 30) in a pattern that cannot always be accounted for by the level of PKC activity. This has led to suggestions of a possible regulatory role for Ca(2+)/calmodulin-dependent protein kinase II as a mediator of some of these effects (30–33). In addition, Ca(2+)/calmodulin itself appears to directly bind and activate the NHE-1 isoform (34, 35). These molecular mechanisms are not fully resolved but clearly differ from osmotic regulation of the exchangers, which is ATP-dependent (36) but does not appear to involve direct phosphorylation of the exchanger, at least in the case of NHE-1 (37). This process may involve other ancillary factors such as G proteins that are independent of the PKA and PKC pathways (37–39). At present, little information is available concerning the stimuli that selectively modulate the individual NHE isoforms and their mechanisms of action (8).

In order to delineate Na+–H+ exchanger regulation by serine/threonine kinases in greater detail, we have stably transfected individual NHE isoforms (NHE-1, NHE-2, and NHE-3) into Chinese hamster ovary cells that are devoid of endogenous exchanger activity (AP-1 cells). We reasoned that a common cellular background should provide a useful model system in which to compare distinct exchangers. The aim of the present study was to test the hypothesis that the NHE isoforms have intrinsic capabilities to respond to PKA and PKC, since previous studies suggested that they are two of the major signaling pathways modulating Na+–H+ exchanger activity in various cell types. The present results support the notion that these isoforms are differentially responsive to these signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials—**Carrier-free 22NaCl (radioactivity, 5 mCi/ml) was obtained from DuPont NEN. Amlodipine was purchased from Sigma, and the amiloride derivative 5-N-(ethyl-N-isopropyl) amlodipine (EIPA) was purchased from Molecular Probes (Eugene, OR). Phorbol 12-myristate 13-acetate (PMA), forskolin, dibutyryl-cAMP, 8-(4-chlorophenylthio)-cAMP (cpt-cAMP), dibutyryl-cGMP, 8-bromo-cGMP, isobutylmethylxanthine, cholera toxin (CTX), chelerythrine chloride, and H-89 were purchased from Biomedical Research Laboratories, Inc. (Plymouth Meeting, PA). A-Minimal essential medium (α-MEM), fetal bovine serum, kana-

mucin sulfate, genicin (G418 sulfate), and trypsine-EDTA were purchased from Life Technologies (Burlington, Canada). Cell culture dishes and flasks were purchased from Becton Dickinson (Fisher Scientific, Montréal, Canada). All other chemicals and reagents used in these experiments were purchased from British Drug House (St. Laurent, Québec) or Fisher Scientific and were of the highest grade available.

**Stock Solutions—**Stock solutions of forskolin (10 mM), PMA (1 mM), chelerythrine chloride (10 mM), H-89 (100 mM), EIPA (100 mM), and amiloride (500 mM) were prepared in dimethyl sulfoxide (Me2SO).

**Cell Culture—**Mutant Chinese hamster ovary cells that are devoid of endogenous Na+–H+ exchanger activity (AP-1 cells) (40) were generated by Dr. S. Grinstein (Hospital for Sick Children, Toronto, Ontario). AP-1 cells stably expressing rat NHE-1, -2, and -3 were used as described previously (6, 7). All cells were maintained in complete α-MEM supplemented with 10% fetal bovine serum, 100 μg/ml kanamycin sulfate, and 25 mM NaHCO3 (pH 7.4) and incubated in a humidified atmosphere of 95% air, 5% CO2 at 37 °C.

For experimentation, the cells were subcultured at 5 × 10^4 cells/well in 24-well plates and grown to confluence. The cells were arrested at the G1/S stage by washing the monolayers with phosphate-buffered saline and incubating in serum-free α-MEM medium for 17–20 h.

**Measurement of Na+ influx—**Na+/H+ exchanger activity was assayed by measuring EIPA- or amiloride-inhibitable 22Na+ influx. Briefly, growth-arrested confluent cell monolayers were washed twice with modified NaCl solution (5 mM NaCl, 5 mM MgCl2, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4). The cells were preincubated in the same solution with various stimulatory or inhibitory agents for the indicated periods of time (see figure legends) at 37 °C. After the preincubation, cells were washed twice with isotonic choline chloride solution (130 mM choline chloride, 1 mM MgCl2, 2 mM CaCl2, 10 mM HEPES-Tris, pH 7.4). 22Na+ uptake was initiated by the addition of choline chloride solution supplemented with the various agents, 1 μCi/ml 22NaCl (carrier-free), 1 mM ouabain, and in the absence or presence of either 100 μM EIPA or 1 mM amiloride (where indicated). At specified time intervals, 22Na+ uptake was terminated by washing the cells 3 times with NaCl stop solution (130 mM NaCl, 1 mM MgCl2, 2 mM CaCl2, 20 mM HEPES-NaOH, pH 7.4). Under the conditions used, initial experiments showed that 22Na+ uptake was linear with time for at least 12 min (data not shown). Therefore, an influx period of 12 min was selected for most studies and represents initial rates of transport.

In experiments where exchanger activity was to be determined from the rate of 22Na+ influx at constant H+ concentration, pH7 was ramped by adding the cells to solutions of high K+ concentration containing the K+ /H+ exchange ionophore nigericin (41). Because at equilibrium [K+]/[H+] = [H+]/[K+], the desired pH was calculated from the imposed [K+] gradient and the extracellular pH (pH7 = 7.5), assuming an intracellular [K+] of 140 mM. Briefly, the monolayers were washed twice with Na+ solution and then preincubated for 15 min in K+ solution (70 mM KCl, 60 mM choline chloride, 1 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 4 mM nigericin, 100 mM bumetanide, 20 mM HEPES-Tris, pH 7.5) at 37 °C. This solution was then replaced with fresh K+ solution supplemented with 1 μCi/ml 22NaCl, 1 mM ouabain, and in the absence or presence of 1 mM amiloride.

To extract the radiolabeled, 0.25 ml of 0.5 N NaOH was added to each well. The wells were washed with 0.25 ml of 5 M HCl. Both the solubilized cell extract and wash solutions were suspended in 5-mL scintillation fluid, and the radioactivity was assayed by liquid scintillation spectroscopy. Protein content was determined using the Bio-Rad DC Protein Assay procedure.

Intracellular pH Measurements—Cells were cultured to confluence on individual glass coverslips pretreated with poly-L-lysine to promote attachment and then loaded with the cell-permeant pH fluorescent dye 2,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethylester (BCECF-AM; Molecular Probes, Eugene, OR). Briefly, 25 μM of BCECF-AM was dissolved in 25 μM MeSO4, to which 12.5 μM of pluronic F-127 (20% in MeSO4, 50 μM pluronic F-127 in final loading solution) was then added. This mixture was added, under intense shaking, to 4 ml of cell culture medium. Cultured monolayers or 4 ml of 1-day-old cultures were immersed in the loading medium for 10 min at room temperature and then rinsed once with cell culture medium to wash out excess BCECF-AM.

BCECF-loaded cells were mounted in the bottom of a laminar flow temperature-controlled chamber (volume, ~350 μL). Silicone rubber was used to complete a water-tight seal. The chamber was mounted on the stage of a Nikon inverted microscope equipped for epifluorescence (Diaphot, Nikon, Tokyo, Japan). The light source was a 75-watt mercury-xenon arc lamp powered by a DC power supply. Excitation light was passed through one of two differential interference filters (440 or 490 nm ± 5 nm) mounted in a turret which could be rotated using a computer-controlled stepping motor. The light was then passed through a 510-nm dichroic mirror and a 40 × Nikon UV-fluor immersion lens with a numerical aperture of 1.3. All fluorescent light passed back through the dichroic mirror and 515-nm bandpass filter to reduce background fluorescence. The emitted fluorescence was detected by the eyepieces or to an intensified charge-coupled device video camera (Micro Photon Devices 2468, Hamamatsu Photonics K.K., Hamamatsu City, Japan). Emitted light at wavelengths between 510 and 530 nm was captured during illumination at each excitation wavelength at the rate of 32 frames/sec. All analyses were performed using a computer-based image analysis system (Fluor-1; Universal Imaging, West Chester, PA).

Twelve frames were averaged to produce a gray scale image, which was corrected on a pixel-by-pixel basis using background images that had been acquired from cell-free areas of the coverslip. Autofluorescence was undetectable, as determined by measuring non-BCECF-loaded cells (<1.5% and <3% of the baseline fluorescence of BCECF-loaded...
cells during excitation with 440 and 490 nm, respectively). For each pair of images, the ratio of the fluorescence intensity at 520 nm during excitation at 490 nm versus the intensity of fluorescence at 520 nm during excitation at 440 nm was calculated, again on a pixel-by-pixel basis. Individual cells were identified using the image of fluorescence during excitation at 490 nm, and one cytosolic area was defined and marked per cell. The ratios for each defined area were stored on a computer disk. The epifluorescence light path was blocked with a shutter between fluorescence measurements to minimize photo-bleaching of the BCECF and cell UV damage. Under this protocol, one ratio image was acquired every 1.4 s. A pseudo-color ratio image as well as a graph showing the ratio for each of the areas of interest was displayed on a color monitor (ECM1311U, Electrohome, Kitchener, Ontario). Stored ratios were imported into a spreadsheet (Lotus 123) where pH calculations were performed.

Calibration of intracellular pH was performed by perfusing the cells with high potassium saline (120 mM potassium gluconate, 10 mM NaCl, 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, 10 mM glucose, and 10 mM HEPES containing 50 μM nigericin (41). The pH of the extracellular saline was varied between pH 6.2 and 8.7. Fluorescence ratios were obtained at each calibration pH (after equilibrium was reached), and a standard curve was generated using MicroCal Origin (MicroCal Software Inc, Northampton, MA) running under Windows 3.1 (Microsoft Corp.) and exported to Lotus 123. Intracellular pH values were then calculated from the experimental fluorescence ratios.

Data Presentation—All data are presented as mean ± standard deviation for the indicated number of observations (n). AII 22Na$^+$ influx experiments were repeated at least 2–4 times in quadruplicate. Intracellular pH measurements were repeated 3–5 times. Data were analyzed by a two-tailed Student’s t test, and differences between test and control values were regarded as statistically significant at p < 0.05.

RESULTS

Involvement of Heterotrimeric GTP-binding Proteins in Regulating the NHE Isoforms—Heterotrimeric G proteins can be segregated into four major classes (Gs, Gi, Gq, and G12). Based on the known effects of hormones such as dopamine, parathyroid hormone, parathyroid hormone-related peptide, and angiotensin II, those G proteins that modulate adenylate cyclase (e.g. Gs and Gi1) and/or phospholipase Cβ (e.g. Gq) are the ones most likely to regulate Na$^+$/H$^+$ exchangers. Nevertheless, information regarding the involvement of G proteins in regulating Na$^+$/H$^+$ exchange activity is limited (38, 39, 42–47), largely because it has been unclear which isoform is being examined in native cells. Hence, it was of particular interest to define the response of specific NHE isoforms to G protein and second messenger agonists.

To detect the general participation of G proteins in regulating NHE isoform activities in intact cells independently of receptors, the effects of AIF$_2$ were examined in stably transfected AP-1 cells individually expressing NHE-1, -2, or -3. AIF$_2$ interacts with G proteins by forming a Go-GDP-AIF$_2$-F$_3$ complex that mimics GTP in a manner that closely resembles that of nonhydrolyzable guanine nucleotide analogues such as GTPyS and, therefore, is a convenient and general means of stimulating G protein-mediated pathways (48). As illustrated in Fig. 1, pretreatment of cells with AIF$_2$ for 60 min to activate G proteins stimulated amiloride-inhibitable 22Na$^+$ uptake by cells expressing NHE-1 or -2 and inhibited uptake into cells expressing NHE-3. No amiloride-inhibitable 22Na$^+$ influx was observed in the parental AP-1 cells (data not shown). Of course, this regulatory pattern represents a composite effect that depends not only on the transacted NHE isoforms but also on the exact cellular complement of G proteins and effectors present in AP-1 cells.

To isolate the actions of a specific class of G proteins, CTX was used since it selectively activates Gs by ADP-ribosylating the α$_s$ subunit near the GTP-binding site and inhibiting GTP hydrolysis (48). The liberated α$_s$ independently stimulates adenylate cyclase activity, and this response can be enhanced or antagonized by the presence of the βγ subunits, depending on the subtype of adenylate cyclase (13, 49). Thus, a cellular response to CTX most likely indicates involvement of the cAMP-PKA pathway. As shown in Fig. 2A, CTX stimulated NHE-1 -2 and inhibited NHE-3 in a concentration-dependent manner, a pattern similar to that observed for AIF$_2$. The effects of CTX were not affected in cells depleted of PKC by overnight incubation (18–24 h) with 200 μM PMA (50) (Fig. 2B) or by 1 μM chelerythrine chloride (51), a highly specific and potent inhibitor of the catalytic domain of PKC (data not shown). However, the effects were abrogated by 100 μM H-89, a highly selective PKA antagonist (52) (Fig. 2B). Thus, these data are consistent with the notion that G proteins linked to the adenylate cyclase-cAMP-PKA pathway are involved in differentially regulating isoforms of the Na$^+$/H$^+$ exchanger. Unfortunately, specific involvement of G$_s$ in regulating the NHE isoforms through the phospholipase Cβ-diacylglycerol-PKC pathway could not be readily assessed due to the absence of a selective agonist for this G protein. Therefore, to further define the signaling pathways that function downstream of G$_s$ and G$_q$, specific activators of PKC and PKA were examined.

Influence of Phorbol Ester and cAMP Activators on 22Na$^+$ Influx by NHE Isoforms—To assess the influence of PKC and PKA on the transport activities of the NHE isoforms, the AP-1 cell transfecants were treated with PMA or forskolin, agents known to stimulate these pathways, respectively. Preliminary concentration-response experiments demonstrated that near maximal effects were achieved with 1 μM PMA and 10 μM forskolin (data not shown); therefore, these concentrations were adopted for subsequent analyses. In the presence of PMA (Fig. 3A), the influx of EIPA-inhibitable 22Na$^+$ was elevated ~78% in cells expressing either NHE-1 or NHE-2, while having a small inhibitory effect (~22%) on NHE-3. Similarly, forskolin treatment resulted in a 96 and 66% stimulation of NHE-1 and NHE-2, respectively. In contrast, the influx of EIPA-inhibitable 22Na$^+$ by cells expressing NHE-3 was substantially depressed by 66% following forskolin treatment. The inactive phorbol ester, 4α-HPMA (1 μM), had no effect on 22Na$^+$ influx by any of

![Fig. 1. Influence of AIF$_2$ on activities of rat Na$^+$/H$^+$ exchanger isoforms stably expressed in Chinese hamster ovary AP-1 cells.](image-url)
Second Messenger Regulation of Rat \( \text{Na}^+ / \text{H}^+ \) Exchanger Isoforms

**Fig. 2.** Influence of cholera toxin on activities of rat \( \text{Na}^+ / \text{H}^+ \) exchanger isoforms stably expressed in AP-1 cells. Confluent AP-1 cell transfectants expressing rat NHE-1 (solid bars), NHE-2 (dotted bars), or NHE-3 (striped bars) were incubated in serum-free medium overnight before assaying for NHE activity. A, prior to \( 22\text{Na}^+ \) influx measurements, the cells were preincubated in isotonic NaCl solution containing increasing concentrations of cholera toxin (CTX; 1–1000 ng/ml) for 1 h. The cells were rapidly washed with Na\(^{-}\)-free, isotonic choline chloride solution and then incubated in choline chloride solution containing \( 1 \mu \text{M} \) CTX (carrier-free), 1 mM ouabain, the varying concentrations of CTX, and either in the absence or presence of 100 \( \mu \text{M} \) EIPA. \( 22\text{Na}^+ \) influx was terminated after a 12-min incubation period. Low levels of background \( 22\text{Na}^+ \) influx that were not inhibitable by 100 \( \mu \text{M} \) EIPA were subtracted from the total influx. NHE activity was defined as EIPA-inhibitable \( 22\text{Na}^+ \) influx and presented as a percentage of control values. Each value is the mean \( \pm \) S.D. (n = 8–14) from two to four experiments. B, to assess the involvement of serine/threonine protein kinases in mediating the effects of CTX, cells were either depleted of PKC activity by overnight incubation (18–24 h) with PMA (200 \( \text{ng} \)) or exposed to the PKA antagonist H-89 (100 \( \mu \text{M} \)) for 1 h in serum-free α-MEM medium prior to CTX treatment. Cells were subsequently preincubated in isotonic NaCl solution containing CTX (1 \( \mu \text{g} / \text{ml} \)) for 1 h and then assayed for NHE isoform activities as described above. Values represent the mean \( \pm \) S.D. (n = 8–16) from two to four experiments. Significant difference from control values was determined by a two-tailed Student’s t test and is indicated by an asterisk (p < 0.05). C, control.

the cell transfectants, suggesting that the cation transport effects mediated by PMA were specific and may be biologically relevant. The biologically inactive forskolin analogue, 19-dideoxyforskolin (10 \( \mu \text{M} \)) also had no effect. Parental AP-1 cells exhibited no detectable EIPA-inhibitable \( 22\text{Na}^+ \) influx under unstimulated conditions or in the presence of PMA or forskolin, consistent with their lack of endogenous NHE activity (data not shown).

In order to confirm that the effect of forskolin was mediated by elevation of cAMP, two additional agents known to increase cAMP levels were tested; cpt-cAMP (0.5 \( \text{mM} \)), a cell-permeable cAMP analog that is relatively resistant to hydrolysis by phosphodiesterases, and 3-isobutyl-1-methylxanthine (1 \( \text{mM} \)), a nonspecific inhibitor of phosphodiesterases. Similar to forskolin, both these agents increased the transport activities of NHE-1 and -2 by approximately 50–100% and depressed the activity of NHE-3 by 50–80% (Fig. 3B). The cell-permeant cGMP analogues dibutyryl-cGMP (1 \( \text{mM} \)) (Fig. 3B) and 8-bromo-cGMP (1 \( \text{mM} \)) (data not shown) had no effect on transport by any of the three isoforms, suggesting that cGMP-dependent protein kinase does not regulate these NHE isoforms, at least when expressed in this cell type. Virtually identical results were obtained with these agents in multiple AP-1 cell lines expressing individual isoforms (data not shown). Therefore, the results were not due to random clonal isolation of AP-1 cell transfectants exhibiting aberrant signaling.

**Fig. 3.** Influence of phorbolester and cAMP agonists on activities of rat \( \text{Na}^+ / \text{H}^+ \) exchanger isoforms stably expressed in AP-1 cells. Confluent AP-1 cell transfectants expressing rat NHE-1 (solid bars), NHE-2 (dotted bars), or NHE-3 (striped bars) were incubated in serum-free medium overnight before assaying NHE activity. Prior to \( 22\text{Na}^+ \) influx measurements, the cells were preincubated for 15 min in isotonic NaCl solution containing either 1 \( \mu \text{M} \) PMA, 1 \( \mu \text{M} \) 4a-PMA, 10 \( \mu \text{M} \) forskolin (F), or 10 \( \mu \text{M} \) 1,9-dideoxyforskolin (1,9-ddF) (A) or 10 \( \mu \text{M} \) forskolin, 0.5 \( \text{mM} \) cpt-cAMP, 1 \( \text{mM} \) isobutylmethylxanthine (IBMX), and 1 \( \text{mM} \) dibutyryl-cGMP (db-cGMP) (B). The cells were rapidly washed with Na\(^{-}\)-free, isotonic choline chloride solution and then assayed for EIPA-inhibitable \( 22\text{Na}^+ \) influx in the continuing presence of the various agents. NHE activity was presented as a percentage of the EIPA-inhibitable \( 22\text{Na}^+ \) influx determined under control conditions. Each value is the mean \( \pm \) S.D. (n = 12–16) from three or four experiments. Significant difference from control values was determined by a two-tailed Student’s t test and is indicated by an asterisk (p < 0.05). C, control.

Stimulation of NHE Isoforms Under pH 7 Clamp Conditions—Forskolin has been reported to induce a small intracellular acidification (ΔpH, < 0.075 units) in some cell types (53). Hence, from a mechanistic viewpoint, it was of interest to determine whether the resultant forskolin-induced increases in NHE-1 and -2 activities were a general consequence of additional H\(^+\) substrate generated by accelerated metabolic activity or were attributable to a signaling event closely associated with the exchangers. Therefore, the ability of forskolin to induce an intracellular acidification in exchanger-deficient AP-1 cells was examined by measuring pH\(_i\) using microfluorometry and a pH-sensitive dye, BCECF. For comparison, the cells were also treated with PMA, which is known not to cause metabolic-induced acidification. The results showed that resting pH\(_i\) was \(~7.0–7.1\) (the tracings were slightly offset for comparative purposes), and neither compound caused any decrease in pH\(_i\) over a 20-min period (Fig. 4A), suggesting that forskolin and PMA stimulation of NHE-1 and -2 was not due to metabolic acidosis. However, the remote possibility remained that small, localized increases in the intracellular H\(^+\) concentration near the exchangers went undetected that, nevertheless, were sufficient to increase \( 22\text{Na}^+ \) influx.
To examine this possibility, pH$_i$ was clamped at 7.2 using K$^+$-nigericin (see "Experimental Procedures") and amiloride-inhibitable $^{22}$Na$^+$ influx was measured into cells exposed to forskolin or PMA. As illustrated in Fig. 4B, forskolin treatment increased NHE-1 and -2 activities by 47 and 36%, respectively. Similarly, PMA treatment stimulated NHE-1 and -2 by 47 and 44%, respectively. The percentage stimulation under pH$_i$-clamped conditions was somewhat lower than with unclamped cells (see Fig. 3). However, this is only an apparent decrease, as the absolute rates of amiloride-inhibitable $^{22}$Na$^+$ influx were higher in control, forskolin-, and PMA-treated cells under pH$_i$-clamped conditions, presumably due to the different buffers used or to differences in pH$_i$ between clamped versus non-clamped cells. Regardless, the results were qualitatively similar using both assays of NHE function and lead to the same conclusion. Both forskolin and PMA enhanced NHE-1 and -2 activities even when the intracellular H$^+$ (substrate) concentration and transmembrane pH gradient were held constant. Their predominant effects were likely mediated by changes in the intrinsic turnover number of the exchangers.

The conclusions drawn from $^{22}$Na$^+$ influx assays were confirmed and extended by measuring NHE activity as the Na$^+$-dependent recovery of pH$_i$ in cells that had been acid-loaded by an NH$_4^+$ prepulse (54). One advantage of this approach over isotope uptakes is the fact that physiological concentrations (130 mM) of Na$^+$ can be used extracellularly when studying the effects of potential stimuli. As illustrated in Fig. 5, for untreated (diluent only) cells expressing NHE-1, -2, and -3, cell acidification was followed by a rapid return to resting cell pH$_i$, and the alkalization was entirely dependent on the presence of Na$^+$, consistent with the involvement of NHE activity. Furthermore, this response was inhibited by amiloride, as previously reported using these cells (36). Treatment of cells with Na$^+$-free choline chloride solution (130 mM choline chloride, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) followed by perfusion in Na$^+$-free choline chloride solution (130 mM choline chloride, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) for 5 min. This treatment typically reduced pH$_i$ to ~6.6 for all cell types. A pH$_i$ recovery from the imposed acid load was triggered by perfusion with isotonic NaCl solution. The data are the mean ± S.E. and are representative of at least three experiments of each kind.

Effect of PKC and PKA Antagonists—The acute stimulation of rat NHE-1 and -2 and inhibition of NHE-3 by PMA implicates PKC; however, PMA has been reported to have effects that are non-PKC-mediated (55–57). Therefore, to evaluate this possibility in AP-1 cells, the influence of PKC antagonists on regulation by PMA was examined. For these studies, attention was focused on NHE-1 and -3 since they represent the two divergent patterns of regulation. As shown in Fig. 6A, down-regulation of PKC by overnight incubation (18–24 h) of the cell transfectants with PMA (200 nM) prevented PMA-induced

**Fig. 4.** Influence of phorbol ester (PMA) and forskolin (F) on resting pH$_i$ in untransfected AP-1 cells and on activities of rat NHE-1 and NHE-2 in AP-1 cells under pH$_i$-clamped conditions. A, untransfected AP-1 cells cultured to subconfluence (~70–80%) on individual glass coverslips were deprived of serum ~5 h and then loaded with the cell-permeant pH fluorescent dye, BCECF/acetoxymethyl ester. The coverslips were rinsed twice with isotonic NaCl solution and then placed in the bottom of a laminar flow-through temperature-controlled chamber. The chamber was sealed and mounted on the stage of a Nikon inverted microscope equipped for epifluorescence and then perfused with isotonic NaCl solution preheated to 37°C. Individual cells within the field of view were selected (n ~ 25 cells), and the fluorescence ratio was continuously monitored as described under "Experimental Procedures." After a 10–15-min equilibration period, the isotonic NaCl perfusate solution was supplemented with either diluent (Me$_2$SO) (●), 1 μM PMA (▲), or 10 μM forskolin (○) (arrow labeled stimulus). At the conclusion of each experiment, the pH$_i$ of cells for each coverslip was calibrated using the K$^+$-nigericin method (41). The resting pH$_i$ of untreated AP-1 cells was ~7.0–7.1, and the data for forskolin and PMA-treated cells were intentionally offset to avoid overlap. Results are the mean ± S.E. and are representative of at least three experiments. B, confluent AP-1 transfectants expressing NHE-1 (filled bars) or NHE-2 (dotted bars) were preincubated for 15 min in a K$^+$-nigericin solution to set pH$_i$ at 7.2 (see "Experimental Procedures" for details) and also contained diluent (Me$_2$SO), 1 μM PMA, or 10 μM forskolin. At the end of this incubation period, the solution was aspirated and replaced with the same solution supplemented with 1 μCi/ml $^{22}$NaCl, 1 mM ouabain in the absence or presence of 1 mM amiloride. Isotope uptake was terminated after 12 min, and the samples were processed as described under "Experimental Procedures." Data are presented as a percentage of the amiloride-inhibitable $^{22}$Na$^+$ influx determined under control conditions. Each value is the mean ± S.D. (n = 12) from three experiments. Significant difference from control values was determined by a two-tailed Student's t test and is indicated by an asterisk (p < 0.05). C, control.

**Fig. 5.** Influence of phorbol ester and forskolin on rates of acid-induced pH$_i$ recovery in AP-1 cell transfectants expressing rat NHE-1, NHE-2, and NHE-3. Subconfluent (~70–80%) AP-1 cell transfectants expressing NHE-1, NHE-2, or NHE-3 were deprived of serum for ~5 h and then loaded with BCECF/acetoxymethyl ester. After mounting the coverslips on a Nikon inverted microscope equipped for epifluorescence, the cells were perfused with isotonic NaCl solution preheated to 37°C. After a 10–15-min equilibration period, the isotonic NaCl perfusate solution was supplemented with control diluent (Me$_2$SO) (●), 1 μM PMA (▲), or 10 μM forskolin (○) for 5 min. These agents were also present in all subsequent solutions throughout the experiment. Cells were then acidified by using the NH$_4^+$ prepulse technique (54). Briefly, cells were acid-loaded for 5 min in NH$_4$Cl solution (25 mM NH$_4$Cl, 105 mM choline chloride, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) followed by perfusion in Na$^+$-free choline chloride solution (130 mM choline chloride, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) for 5 min. This treatment typically reduced pH$_i$ to ~6.6 for all cell types. A pH$_i$ recovery from the imposed acid load was triggered by perfusion with isotonic NaCl solution. The data are the mean ± S.E. and are representative of at least three experiments of each kind.
stimulation of NHE-1 and inhibition of NHE-3, while having no effect on the actions of forskolin. These observations were corroborated by pretreating NHE-1 and -3 transfectants with the PKC inhibitor chelerythrine chloride (1 μM). As illustrated in Fig. 6B, this compound suppressed PMA-induced stimulation and inhibition of 22Na⁺ influx by NHE-1 and -3, respectively. Thus, the results indicate that the mechanism of PMA activation of NHE-1 and inhibition of NHE-3 in AP-1 cells is mediated through the PKC pathway. Furthermore, inhibition or depletion of PKC did not impair forskolin-mediated stimulation and inhibition of NHE-1 and -3, respectively, suggesting that its actions are mediated by an independent pathway, presumably PKA, that does not involve downstream activation of PKC.

If the antithetical effects of forskolin on NHE-1 and -3 were mediated through activation of PKA, both responses should be inhibited by antagonists of PKA, such as H-89. Indeed, the stimulation of NHE-1 and the inhibition of NHE-3 were both attenuated in the presence of H-89, strongly implicating PKA in this process (Fig. 7). It is interesting that NHE-1 activity in the presence of H-89 alone was significantly repressed by 75%, suggesting that a substantial portion of its basal activity was dependent on basal PKA activity. Opposite results, though quantitatively less dramatic, were obtained for NHE-3 activity, which showed a marginal 15% stimulation in the presence of H-89 alone.

Fig. 6. Effect of PKC inhibition on phorbol ester (PMA) and forskolin (F) regulation of rat NHE-1 (filled bars) and NHE-3 (striped bars) in AP-1 cells. AP-1 cell transfectants expressing either rat NHE-1 or NHE-3 cells were grown to confluence in 24-well plates. Prior to 22Na⁺ influx measurements, the cells were depleted of PKC activity by overnight preincubation with 200 nm PMA (A) or by exposure to diluent or the PKC antagonist chelerythrine chloride (1 μM) for 1 h in serum-free α-MEM medium (B). In A, cells were subsequently preincubated for 15 min in isotonic NaCl solution containing diluent, 1 μM PMA, or 10 μM forskolin, whereas in B, cells were treated with the different agents either alone or in the combined presence of chelerythrine chloride. Cells were then assayed for NHE isoform activities as described under “Experimental Procedures.” Values represent the mean ± S.D. (n = 12) from three experiments. Significant difference from control values was determined by a two-tailed Student’s t test and is indicated by an asterisk (p < 0.05). C, control.

Fig. 7. Effect of PKA inhibition on forskolin-mediated regulation of rat NHE-1 (filled bars) and NHE-3 (striped bars) in AP-1 cells. Confluent AP-1 cell transfectants expressing either rat NHE-1 or NHE-3 were preincubated in the absence or presence of the PKA antagonist H-89 (100 μM) for 1 h in serum-free α-MEM medium. Cells were then incubated for an additional 15 min in isotonic NaCl solution containing either diluent or 10 μM forskolin (F) in the absence or presence of H-89 prior to measurements of initial rates of amiloride-inhibitable 22Na⁺ influx. Each value is the mean ± S.D. (n = 12) from three experiments. Significant difference from control values was determined by a two-tailed Student’s t test and is indicated by an asterisk (p < 0.05). C, control.

DISCUSSION

Studies defining the regulation of individual NHE isoforms by distinct intracellular signaling pathways have only recently been undertaken. The results from this study demonstrate that activation of the PKA or PKC pathway can lead to stimulation of NHE-1 and -2 as well as inhibition of NHE-3 when the exchangers are stably expressed in AP-1 cells.

Regulation of the Na⁺/H⁺ exchanger by numerous hormones and growth-promoting agents is well documented (reviewed in Refs. 5 and 12). Since many of these agents bind to cell surface receptors that ultimately activate distinct serine/threonine protein kinases, it is likely that heterotrimeric G proteins play an essential intermediary role in the transmembrane signaling events that lead to altered Na⁺/H⁺ exchanger activity. Busch et al. (47) have recently shown that microinjection of GTPγS or purified Gβγ subunits of transducin into Xenopus laevis oocytes stimulated native Na⁺/H⁺ exchanger activity by the PKA or PKC pathways, respectively. However, it is unclear whether both pathways activated the same or distinct isoforms of the exchanger in oocytes. Using human embryonic kidney 293 cells as hosts, transient expression of constitutively activated mutants of Gαq, and Gα13 enhanced Na⁺/H⁺ exchanger activity, whereas Gαq and Gα12 were without effect (39). Interestingly, while Gαq appeared to exert its effects through the phospholipase Cβ pathway, Gα₁₂ competent without modifying intracellular levels of inositol phosphate and cAMP, suggesting the involvement of a novel signaling pathway. In a comparable study using COS-1 cells, transient expression of activated Gαq, Gα₁₂, and Gα₁₃ also stimulated the Na⁺/H⁺ exchanger, while, on the contrary, Gαq inhibited its activity and Gα₁₃ was without effect (45). Depleting cells of PKC activity abolished the enhancement caused by Gαq and Gα₁₂, but did not affect the stimulation mediated by Gα₁₃. Thus, G proteins such as Gα₁₂ appear to activate the Na⁺/H⁺ exchanger by a distinct pathway that is independent of PKA and PKC. Indeed, Gα₁₃ has recently been shown to activate the J un kinase/stress-activated protein kinase pathway (58), suggesting that this kinase may be linked to the regulation of the Na⁺/H⁺ exchanger. Again, however, it is unclear which isoforms of the Na⁺/H⁺ exchanger are being regulated in these cell types. The results from our study partially clarify this issue by showing that specific activation of Gαq (by cholera toxin) stimulated the activities of NHE-1 and NHE-2 and inhibited that of NHE-3 through a signaling pathway involving PKA. Further studies using this heterologous expression system are currently ongoing to define the involvement of other G proteins.
Previous studies have convincingly demonstrated that the phospholipase C-diacylglycerol-PKC pathway constitutes a major signaling route for activation of the ubiquitous NHE-1 isoform of the exchanger. Stable expression of human (59) and rabbit (8) NHE-1 in fibroblastic cells (PS120) has shown that this isoform is rapidly activated following acute cell stimulation by phorbol esters as well as by growth factors and other mitogens. Our results with rat NHE-1 expressed in AP-1 cells confirm these results; however, the precise molecular mechanism remains unclear.

Stimulation of NHE-1 by phorbol esters and other growth-promoting agents was initially attributed to an increase in the phosphorylation of a common set of tryptic peptide fragments in the C-terminal region of the exchanger (15, 59). These data implied that the different agonists, which stimulated diverse signaling pathways, ultimately transmitted their signals to a common protein kinase that phosphorylated and activated the exchanger. However, subsequent studies revealed that deletion of this region (amino acids 635–815) only partially impaired (50%) activation, whereas removal of another upstream region (amino acids 567–635), which does not contain any of the phosphorylation sites, completely abolished activation by several growth-promoting agents (60). The involvement of multiple regulatory regions to account for the stimulation of NHE-1 by diverse agents has also been supported by studies of Winkel et al. (61), who demonstrated that microinjection of a polyclonal antibody raised against amino acids 658–815 of NHE-1 ablated the stimulation mediated by endothelin-1 and α-thrombin but was ineffective in preventing activation induced by phorbol ester and hyperosmotic medium. These data suggested that other mechanisms in addition to direct phosphorylation of NHE-1 may play an important role in regulating its activity. One possible mechanism that has been proposed is the participation of exchanger-associated regulatory factors that themselves may also be targets of protein kinases. In support of this argument, a 24-kDa protein has recently been found to associate with NHE-1, although its functional significance has not yet to be defined.2

In contrast to PKC-mediated activation of NHE-1, evidence supporting a role for cAMP in the regulation of NHE-1 is rather sparse and contradictory, and this has lead to the general view that this isoform is not responsive to this second messenger. Previous studies have shown that human (53) and rabbit (8) NHE-1 expressed in PS120 fibroblastic cells are unresponsive to cAMP analogues. However, a subsequent study showed that when human NHE-1 was stably transfected into opossum kidney (OK) cells, its activity was inhibited by activation of PKA (induced by forskolin) or PKC (induced by phorbol ester), suggesting possible cell-specific regulatory effects (17). In contrast, primary rat hepatocytes (22) and murine macrophages (23) showed significant cAMP-induced stimulation of Na+/H+ exchanger activity. Subsequent investigations have revealed that these tissues express only the NHE-1 isoform (2, 3, 62). More recent studies have found that the rat osteoblastic cell line, UMR-106, also expresses NHE-1 exclusively and that it is cAMP-activatable (57). Consistent with these studies, the data presented herein show that rat NHE-1 stably expressed in AP-1 cells is also stimulated by agonists that increase cAMP accumulation, thereby suggesting that this stimulatory response is an intrinsic property of this isoform.

In addition to rat NHE-1, the trout red cell also expresses a Na+/H+ exchanger, called βNHE, that is phorbol ester- and cAMP-activatable in PS120 fibroblasts and has a primary structure with highest identity to that of mammalian NHE-1 (53).

The trout βNHE contains two optimal consensus sites for phosphorylation by PKA (R(K/R)X(S/T*) at Ser641 and Ser648), which, when simultaneously mutated to Gly, partially reduced (by −72%) the ability of cAMP to activate the exchanger (63). This residual cAMP-activatable activity was found to require amino acids 559–661 that may contain cryptic PKA sites that have yet to be identified or, alternatively, may interact with cAMP/PKA-regulated accessory factors. Interestingly, mutation of the two serine residues did not alter the capacity of βNHE to be induced by phorbol ester, suggesting that the actions of PKC are not convergent with those of PKA and are mediated elsewhere in the exchanger. Furthermore, these results suggested that the absence of cAMP regulation of human and rabbit NHE-1 in the same cell line (i.e. PS120) is likely not a consequence of a dysfunctional PKA pathway but perhaps, as suggested above, due to the absence of other cell-specific cAMP/PKA-regulated factors that interact with NHE-1. It is also worth noting that while rat NHE-1 contains several putative PKC consensus sequences ((R/K)_{1–3}(S/T*)X_{6–0}(R/K)_{1–3}) in its C-terminal region (2), it does not contain a classical consensus site for PKA. However, since there is overlap in consensus sequence determinants among protein kinases (64), one cannot exclude the potential for PKA phosphorylation of NHE-1. In summary, while it is difficult at the present time to reconcile the variable regulation of NHE-1 by increasing cAMP, several factors operating independently or in combination may account for these observations, such as cell-specific differences in the expression of signaling components, putative exchanger-associated regulatory factors, or perhaps species variation. Further studies are in progress to define the molecular mechanism by which NHE-1 is regulated by PKA.

Unlike NHE-1, much less is known about second messenger regulation of NHE-2. In a SV-40-transformed rabbit S2 proximal tubule (RKPC-2) cell line, native NHE-2, which appears to reside on the apical membrane, was inhibited by 8-bromo-cAMP, whereas it was stimulated by PMA (65). However, heterologous expression studies have shown that rabbit NHE-2 in PS120 fibroblastic cells was similarly activated by phorbol esters as well as serum but was unresponsive to cell-permeant cAMP analogues (8). The results from the present study partially corroborate these results by showing that rat NHE-2 in AP-1 cells is also stimulated by phorbol ester but differ in that it is enhanced by cAMP analogues as well. The variable responsiveness of NHE-2 to cAMP probably depends on the cell type. Unlike NHE-1, the C-terminal cytoplasmic domain of rat and rabbit NHE-2 contains several classical consensus sequences for phosphorylation by PKA as well as PKC. The question of whether kinase action is mediated through phosphorylation of these sites or possibly via cell-specific exchanger-associated regulatory factors awaits future studies.

In contrast to NHE-1 and NHE-2, the NHE-3 isoform of the Na+H+ exchanger in AP-1 cells is unique in that it exhibits decreased rates of transport in response to G protein and second messenger agonists of the PKA and PKC pathways. An identical pattern of regulation is also observed in AP-1 cells when this isoform is exposed to hyperosmotic medium (36). This distinctive regulation precisely mimics that observed for the endogenous, apically targeted NHE-3 isoform in renal proximal tubule opossum kidney cells where hyperosmolarity (66) and agonists of the PKA and PKC signaling pathways (67, 68) inhibit its activity. Thus, AP-1 cells provide a useful model for investigating the mechanism by which these diverse stimuli converge to inhibit NHE-3.

Analogous results have partially been obtained using rabbit NHE-3 stably expressed in fibroblastic cells (PS120), which was inhibited by acute exposure to PMA but unresponsive to

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elevated cAMP, (8). On the other hand, the rabbit renal Na+/H+ exchanger in isolated brush border membrane vesicles (presumably NHE-3) was inhibited by PKA (19). Thus, cAMP-mediated regulation of NHE-3 appears to be cell-specific.

More recent structural analyses by us suggest that a region between amino acids 579 and 684 of rat NHE-3 reveals the presence of potential consensus sequences for PKA as well as for PKC within or in close proximity to this region, which nevertheless similarly influence exchanger activity. However, the molecular signaling events that occur between activation of these kinases and the responses of NHE-3 are unclear. For example, it is unknown whether these protein kinases mediate their effects by direct phosphorylation of NHE-3 or indirectly via phosphorylation-dependent ancillary proteins. With regard to the latter, there is some in vitro evidence that PKA-mediated inhibition of the rabbit renal apical Na+/H+ exchanger requires the involvement of a regulatory protein that is separate from the kinase and transporter (70). Cell-specific expression of these factors could account for the variable responsiveness of NHE-3 to individual protein kinases. Further studies are ongoing to confirm this hypothesis and identify the precise molecular mechanisms involved.

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Plasma Membrane Na\(^+\)/H\(^+\) Exchanger Isoforms (NHE-1, −2, and −3) Are Differentially Responsive to Second Messenger Agonists of the Protein Kinase A and C Pathways

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