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Am J Physiol Cell Physiol
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Acute inhibition of brain-specific Na^+/H^+ exchanger isoform 5 by protein kinases A and C and cell shrinkage

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Attaphitaya, Surat, Keith Nehrke, and James E. Melvin. Acute inhibition of brain-specific Na^+/H^+ exchanger isoform 5 by protein kinases A and C and cell shrinkage. *Am J Physiol Cell Physiol* 281: C1146–C1157, 2001.—Little is known of the functional properties of the mammalian, brain-specific Na^+/H^+ exchanger isoform 5 (NHE5). Rat NHE5 was stably expressed in NHE-deficient PS120 cells, and its activity was characterized using the fluorescent pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein. NHE5 was insensitive to ethylisopropyl amiloride. The transport kinetics displayed a simple Michaelis-Menten relationship for extracellular Na^+ (apparent $K_{Na} = 27 \pm 5$ mM) and a Hill coefficient near 3 for the intracellular proton concentration with a half-maximal activity at an intracellular pH of 6.93 ± 0.03. NHE5 activity was inhibited by acute exposure to 8-bromo-cAMP or forskolin (which increases intracellular cAMP by activating adenylate cyclase). The kinase inhibitor H-89 reversed this inhibition, suggesting that regulation by cAMP involves a protein kinase A (PKA)-dependent process. In contrast, 8-bromo-cGMP did not have a significant effect on activity. The protein kinase C (PKC) activator phorbol 12-myristrate 13-acetate inhibited NHE5, and the PKC antagonist chelerythrine chloride blunted this effect. Activity was also inhibited by hyperosmotic-induced cell shrinkage but was unaffected by a hyposmotic challenge. These results demonstrate that rat brain NHE5 is downregulated by activation of PKA and PKC and by cell shrinkage, important regulators of neuronal cell function.

pH regulation; amiloride; sodium-proton exchange; sodium/hydrogen

SODIUM/HYDROGEN EXCHANGER isoform 5 (NHE5) is a member of the mammalian family of integral membrane proteins that are involved in the regulation of intracellular pH (pH_i), cell volume, and electrolyte transport (reviewed in Refs. 14, 30, 44, and 52). Other known members of this family include four additional plasma membrane isoforms (NHE1–NHE4; see Refs. 32, 36, and 45) and a mitochondrial isoform NHE6 (28). The deduced amino acid sequence and predicted membrane organization of NHE5 is similar to other members of the Na^+/H^+ exchanger gene family, being most like NHE3 (2, 3). However, in contrast to the epithelial NHE3, high-level expression of NHE5 mRNA is restricted exclusively to the brain (2, 20), suggesting that this isoform performs a specialized role in this tissue.

Individual NHE isoforms have unique functional properties including different exchange kinetics, pharmacological characteristics, cellular localization, and tissue expression (2, 3, 13, 30, 32, 39, 44, 45, 52). The regulation of the different NHE isoforms by second messengers is distinct as well. Two major signaling pathways often involved in regulating the activity of Na^+/H^+ exchangers include the serine/threonine kinases protein kinase A (PKA) and protein kinase C (PKC) (18, 23, 42, 47, 53). The unique sensitivities of the different isoforms to phosphorylation reside in regulatory domains located within the cytoplasmic carboxy terminus, the region where Na^+/H^+ exchangers differ most significantly in their primary sequences. Deletion analysis and domain swapping experiments have confirmed that the carboxy terminus contains the elements sensitive to second messenger regulation (12, 24, 51).

Most tissues express multiple NHE isoforms, and the brain is no exception (2, 20, 32, 45). This makes it difficult, if not impossible, to functionally isolate and characterize NHE5 in native cells. Therefore, the present study investigates the functional properties of rat NHE5 by stable expression of its cDNA in PS120 cells lacking Na^+/H^+ exchanger activity. NHE5 activity was compared with the Na^+/H^+ exchanger activity in PS120 cells expressing NHE1 or NHE3. The functional properties and ethylisopropyl amiloride (EIPA) sensitivity of NHE5 were qualitatively most like NHE3. Furthermore, the inhibition of NHE5 activity in response to cell shrinkage, PKA, and PKC stimulation suggests that the regulation of this brain-specific Na^+/H^+ exchanger may be critical to the activity of neurons in the central nervous system.

MATERIALS AND METHODS

Materials. 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM, carboxyseminaphthorhodafluor (SNARF)-1-AM, and nigericin were from Molecular Probes (Eugene, OR).

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Dulbecco's modified Eagle's medium (DMEM) and G418 were from GIBCO BRL (Grand Island, NY). Phorbol 12-myristate 13-acetate (PMA), 4α-PMA, forskolin, 1,9-dideoxyforskolin (1,9-DDF), 8-bromo-cAMP (8-Br-cAMP), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), and chelerythrine chloride were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). All other reagents were purchased from Sigma (St. Louis, MO). Stock solutions of forskolin, 1,9-DDF, PMA, 4α-PMA, chelerythrine chloride, H-89, and EIPA were prepared in DMSO. 8-Br-cAMP and 8-Br-cGMP were dissolved in deionized, distilled H_2O.

Transfection and stable expression of rat NHE5. The PS120 cells, which lack endogenous Na^+/H^+ exchange activity, were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (50 U/ml)/streptomycin (50 μg/ml). Rat NHE1, NHE3, and NHE5 cDNAs were inserted into the pCMV/SEAP vector (Clontech, Palo Alto, CA), and stable expression was established in NHE-deficient PS120 cells as previously described (2). Cells were selected for stable expression with G418 (1,000 U/ml) and further selected for expression of Na^+/H^+ exchanger activity by the "H^+ killing" method (43). Transfected cells were maintained in medium containing G418, and the H^+ killing procedure was repeated every 4–5 days to eliminate revertants.

RNA isolation and RT-PCR of NHERF and ezrin. Total RNA was prepared from confluent 100-mm plates of culture cells or from 500 mg of hamster kidney using TRIzol reagent (Life Technologies, Rockville, MD). Total RNA (1 μg) was then reverse-transcribed with an oligo(dT) primer using Stratagene's RT-for-PCR kit (La Jolla, CA) as recommended. Five microliters of the diluted first-strand cDNA was subjected to PCR in a final volume of 25 μl that contained 10 mM Tris·HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.08% Nonidet P-40, 200 μM of each dNTP, and 0.4 μM of one of the amplimer pairs described below. Each reaction was brought to 94°C for 1 min, then to 72°C while 0.3 μl of *Taq* polymerase was added, and then cycled at 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s. After the appropriate number of cycles, the reactions were incubated at 72°C for 5 min. The following amplimer pairs were generated from mouse sequences: *1*) Na^+/H^+ exchanger regulatory factor (NHERF): 5′-AGCAATGGAGAGATACAGAAGG-3′ and 5′-TAAGGTGAGGGAAGAACAGG-3′ and *2*) ezrin: 5′-TCACACAGAAGCTCTTCTTCC-3′ and 5′-AGATGTTCCTGATCTCACTCC-3′. The NHERF and ezrin reactions were cycled 35 times (ham-

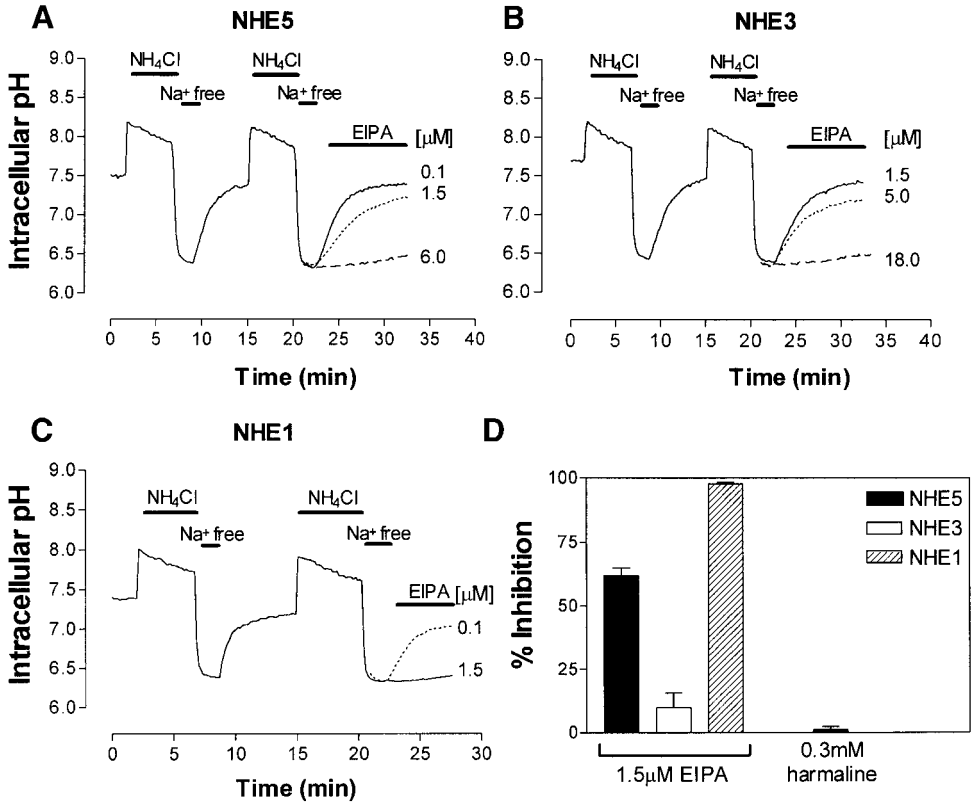

Fig. 1. Inhibition of Na^+-dependent pH recovery of rat Na^+/H^+ exchanger isoform (NHE)5, NHE3, and NHE1 by the amiloride analog ethylisopropyl amiloride (EIPA). PS120 fibroblast cells expressing either rat NHE5, NHE3, or NHE1 were grown on glass coverslips and loaded with the pH-sensitive dye by incubation with the acetoxymethyl ester of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; as described in MATERIALS AND METHODS). To compare Na^+/H^+ exchanger activity of the different NHE isoforms, a paired NH_4^+ prepulse protocol was used in which the same cells were acid loaded, and, following recovery, acid loaded a second time in the presence of EIPA. Representative traces illustrating inhibition of Na^+-dependent recovery by EIPA are shown for NHE5 (*A*), NHE3 (*B*), and NHE1 (*C*). Near total inhibition (>95%) was obtained at EIPA concentrations of 6, 18, and 1.5 μM for NHE5, NHE3, and NHE1, respectively. *D*: relative percent inhibition of the various isoforms at 1.5 μM EIPA. Additionally, the insensitivity of NHE5 to 0.3 mM harmaline is shown. Recovery rates (ΔpH_i/min) were determined from the initial linear portion following the readdition of extracellular Na^+. Rate calculations from the first pulse were compared with the second pulse in the presence of inhibitor. Values represent the means ± SE ($n \geq 30$) from 3 separate experiments.

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ster templates), and the reaction products were separated by size on a 2% agarose gel. cDNA (Qiagen, Chatsworth, CA) was prepared and used directly for cycle DNA sequencing with ABI BigDye terminator mix (Foster City, CA) and thermostable DNA polymerase on an MJResearch autosequencer (Watertown, MA). The reactions were run by the University of Rochester Core Nucleic Acids Facility.

pH_i measurements. The pH_i of individual cells plated on glass coverslips (<50% confluent) was monitored using the pH-sensitive dye BCECF on the microscope stage of an imaging platform (Axon Instruments, Foster City, CA) as previously described (27). BCECF-containing cells were acid loaded using the NH_4Cl prepulse technique (35) to monitor the Na^+-dependent recovery of pH_i. The advantage of this approach is that physiological concentrations of extracellular Na^+ are used to investigate the effects of stimulation. Briefly, coverslips were superfused with a physiological salt solution containing 60 mM NH_4Cl (NaCl was replaced by NH_4Cl) for 10 min and then switched to a Na^+-free salt solution (except where indicated) to produce an acid load. Approximately 3 min later, extracellular Na^+ was restored to initiate Na^+/H^+ exchanger-mediated pH_i recovery. The physiological salt solution contained (in mM) 135 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 0.33 NaH_2PO_4, 10 glucose, 20 HEPES, 1.2 $CaCl_2$, and 0.8 $MgSO_4$, and the pH was adjusted to 7.4 with Tris base. Na^+ was replaced by *N*-methyl-D-glucamine (or choline chloride where indicated in the figure legends). In experiments to test the regulation of exchanger activity by changes in osmolarity, 55 mM NaCl was removed (hypotonic solution; zero sucrose) and replaced with either 110 mM sucrose (isotonic solution) or with 320 mM sucrose (hypertonic solution). The osmolality (mosmol/kgH_2O) of all solutions was determined using a vapor pressure osmometer (Wescore 5500; Logan, UT). The pH-sensitive dye SNARF-1 was used to test the sensitivity of NHE5 activity to harmaline, a Na^+/H^+ exchange inhibitor that interferes with the BCECF fluorescence signal.

Calibration of the pH_i signal was accomplished by the high potassium-nigericin technique (40). Recovery rates (ΔpH_i/min) of the fluorescence traces were determined from the initial linear portion following the readdition of extracellular Na^+.

To directly compare Na^+/H^+ exchanger activity under control and experimental conditions, a paired NH_4^+ prepulse protocol was used in which the same cells were acid loaded, and, then, following recovery ("control" rate), acid loaded a second time under experimental conditions (e.g., in the presence of activators, inhibitors, or different cations). In prelim-

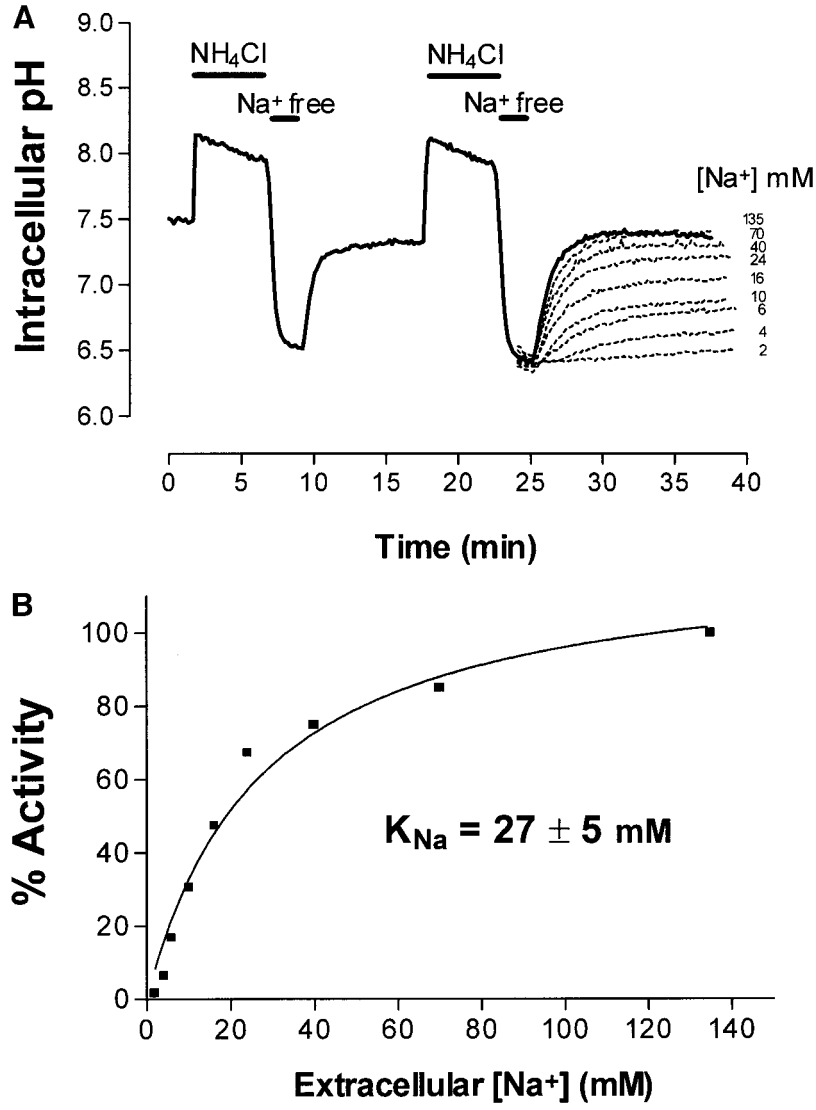

Fig. 2. Extracellular Na^+ dependence of rat NHE5. PS120 fibroblast cells expressing NHE5 were acid loaded using the NH_4^+ prepulse technique (as described in MATERIALS AND METHODS). *A*: Na^+ dependency of the intracellular pH recovery was determined by varying the extracellular [Na^+]. Recovery in 2, 4, 6, 10, 16, 24, 40, and 70 mM Na^+ are overlaid with a continuous double pulse at 135 mM Na^+. Na^+ was replaced with choline chloride. Each trace is the average result from $n > 30$ cells. *B*: relative recovery rates (expressed as percentage of recovery at 135 mM Na^+) were plotted against the external [Na^+]. The extracellular Na^+ kinetics of NHE5 followed a hyperbolic Michaelis-Menten curve with an apparent (affinity constant) $K_{Na} = 27 \pm 5$ mM. Values represent the means of $n \geq 30$ cells from 3 different experiments.

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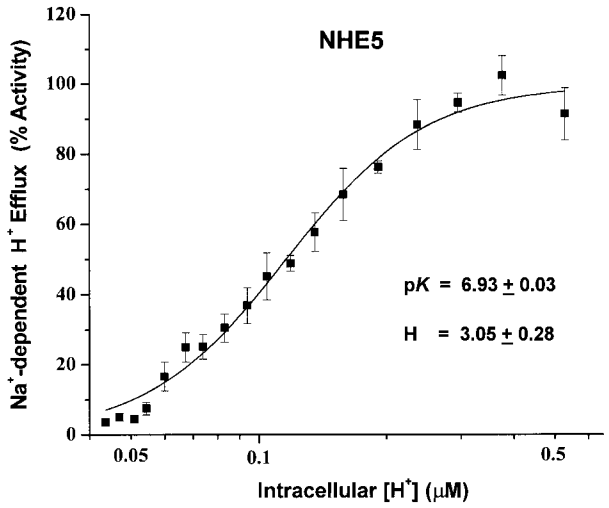

Fig. 3. Na^+-dependent H^+ efflux of rat NHE5 as a function of intracellular $[H^+]$. PS120 fibroblast cells expressing NHE5 were acid loaded using the NH_4^+ prepulse technique, and the intracellular H^+ dependency of the pH recovery was determined over the range of intracellular pH 6.0–7.4, as described in MATERIALS AND METHODS. The apparent affinity constant (pK) and the Hill coefficient (H) were calculated from the Hill equation $\{y = y_{max}/[1 + (pK/[H^+]_i)^H]\}$, where y is the normalized Na^+-dependent rate of H^+ efflux, y_{max} is the maximum rate of H^+ efflux, pK is the half-maximal activity, and H is the Hill coefficient (Origin 6.1). Values represent the average of 6 experiments, each consisting of $n \geq 12$ cells/experiment.

inary experiments, it was determined that the initial recovery rates were not significantly different in two successive control experiments in the same cells (1st pulse: 0.511 ± 0.023 pH_i U/min; 2nd pulse: 0.474 ± 0.023 pH_i U/min, n = 40).

To determine the Na^+/H^+ exchanger activity as a function of the intracellular H^+ concentration, Na^+-dependent pH_i recovery from an acid load was fit to a single exponential function, and the rate of change of pH at a given pH_i was obtained from the derivative of the exponential at that pH (Origin 6.1 software; OriginLab, Northampton, MA). The rate of Na^+/H^+ exchanger activity (hydrogen ion efflux rate in micromolar H^+/s) was then obtained by multiplying the rate of change in pH by the cellular buffering capacity (9).

Data presentation. All data are reported as means ± SE for the indicated number of cells (n). Each experiment was repeated three times on different days (3 different coverslips each day for a total of 9 coverslips/experiment) unless otherwise indicated. Data were analyzed by a two-tailed Student's t-test, and differences between test and control values at $P < 0.05$ were considered statistically significant.

RESULTS

Kinetic properties of rat NHE5. To verify that the functional properties of rat NHE5 are similar to human NHE5 (39) under "physiological" conditions (i.e., in a high external Na^+ concentration), we stably expressed a cDNA containing its open reading frame in NHE-deficient PS120 cells and characterized the resulting Na^+/H^+ exchanger activity using the fluorescent pH-sensitive dye BCECF. For comparative purposes, similar experiments were performed with PS120 cells expressing rat NHE1 and NHE3, the properties of which have been previously described (33, 49). pH_i recovery from an acid load was not observed in nontransfected PS120 cells (data not shown, see Ref. 2). In contrast, an intracellular Na^+-dependent pH recovery was activated in acid-loaded cells transfected with NHE1, NHE3, or NHE5 (Fig. 1). The Na^+-dependent pH recovery observed in NHE5-expressing cells was inhibited by the amiloride analog EIPA, but this block required relatively high concentrations of antagonist (Fig. 1, *A*, and as summarized in *D*). Previous studies have shown that rat Na^+/H^+ exchangers display distinct sensitivities to EIPA (33, 49). Like rat NHE3 (Fig. 1*B*), NHE5 was substantially more resistant than NHE1 to this inhibitor (Fig. 1*C*). These results verify that under the physiological conditions of the current studies, the EIPA sensitivity of NHE1 ≫ NHE5 > NHE3. Rat NHE1, NHE2, and NHE3 are also blocked by harmaline, an inhibitor of Na^+/H^+ exchange unrelated to amiloride: $K_{½}$ = 0.14, 0.33, and 1 mM, respectively (29, 31, 49). Harmaline, up to 0.3 mM, failed to

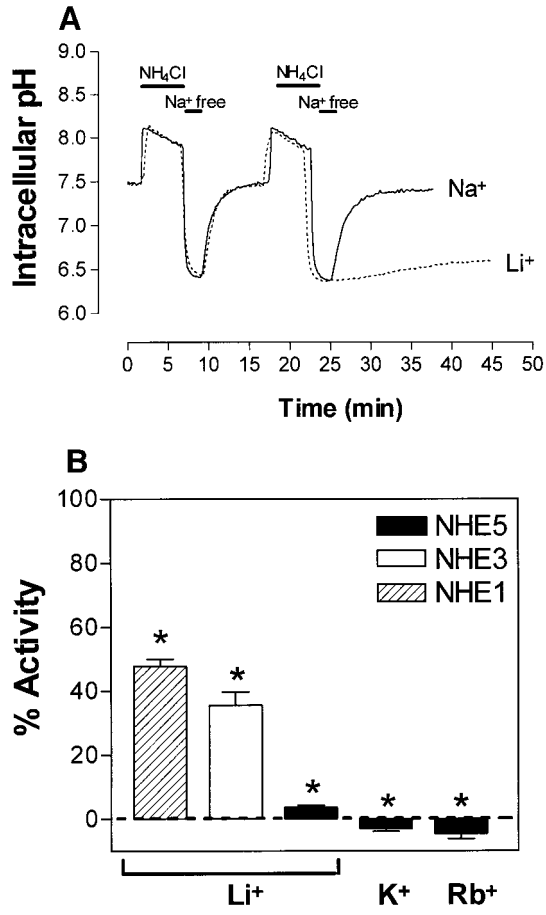

Fig. 4. Extracellular cation selectivity of rat NHE5. PS120 fibroblast cells expressing either NHE5, NHE3, or NHE1 were acid loaded using the NH_4^+ prepulse technique (as described in MATERIALS AND METHODS). *A*: overlay of NHE5 activity in the continuous presence of 135 mM Na^+ (solid line) contrasting the minimal recovery in the presence of 135 mM Li^+ during the second pulse (dotted line). Each trace is the average result from $n > 30$ cells. *B*: summary of the relative recovery rates of NHE5, NHE3, and NHE1 in the presence of 135 mM Li^+. In addition, the inability of NHE5 to transport K^+ and Rb^+ is shown. Values represent means ± SE of $n \geq 30$ cells from 3 different experiments. *Significant differences from the control.

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inhibit NHE5 activity (Fig. 1*D*). We used SNARF-1 for this latter experiment because this pH-sensitive dye is resistant to fluorescence interference by harmaline at the concentrations used.

The Na^+ dependency of NHE5 was determined by monitoring the initial pH recovery rate in varying concentrations of external Na^+ (Fig. 2*A*). The external Na^+ concentration dependence conformed to simple Michaelis-Menten kinetics (Fig. 2*B*). The line was fit to the data using nonlinear regression analysis (GraphPad PRISM, San Diego, CA) that gave an apparent affinity constant (K_{Na}) for NHE5 of 27 ± 5 mM, similar to the 18.6 mM K_{Na} found for human NHE5 (39). The transport activity of rat NHE5 as a function of the intracellular H^+ concentration (Fig. 3) was determined by calculating the rate of hydrogen ion efflux over the range of pH_i 6.0 to 7.4. The half-maximal activation value was pH 6.93 ± 0.03 (n = 6), more alkaline than determined for human NHE5 (pH 6.43) (39). The Hill coefficient (H) for the intracellular proton concentration was >1 (H = 3.05 ± 0.28), indicating the presence of multiple intracellular H^+ binding sites. In contrast, human NHE5 exhibited a first-order dependence on the intracellular H^+ concentration (39). Comparable with previous studies (23, 29), we found that the apparent affinity constants for NHE1 and NHE3 were acidic (NHE1: pK = 6.78 ± 0.07; NHE3: pK = 6.73 ± 0.14) and that these exchangers were characterized by a greater than first-order dependence on the intracellular H^+ concentration (NHE1: H = 3.83 ± 0.44; NHE3: H =2.13 ± 0.20).

The cation selectivity of the different Na^+/H^+ exchanger isoforms has been previously investigated. Li^+ was found to substitute for Na^+ (or H^+) on isoforms NHE1 through NHE4 (13, 17, 49), whereas only NHE4 transported K^+ (13). Figure 4*A* shows that Li^+ was transported by rat NHE5, but very poorly compared with Na^+. In agreement with previous studies, Li^+ was also transported on NHE1 and NHE3 at a slower rate compared with Na^+ (Li^+ transport rate: NHE1 > NHE3 ≫ NHE5; Fig. 3*B*). As summarized in Fig. 4*B*, neither K^+ nor Rb^+ were transported by NHE5. More-

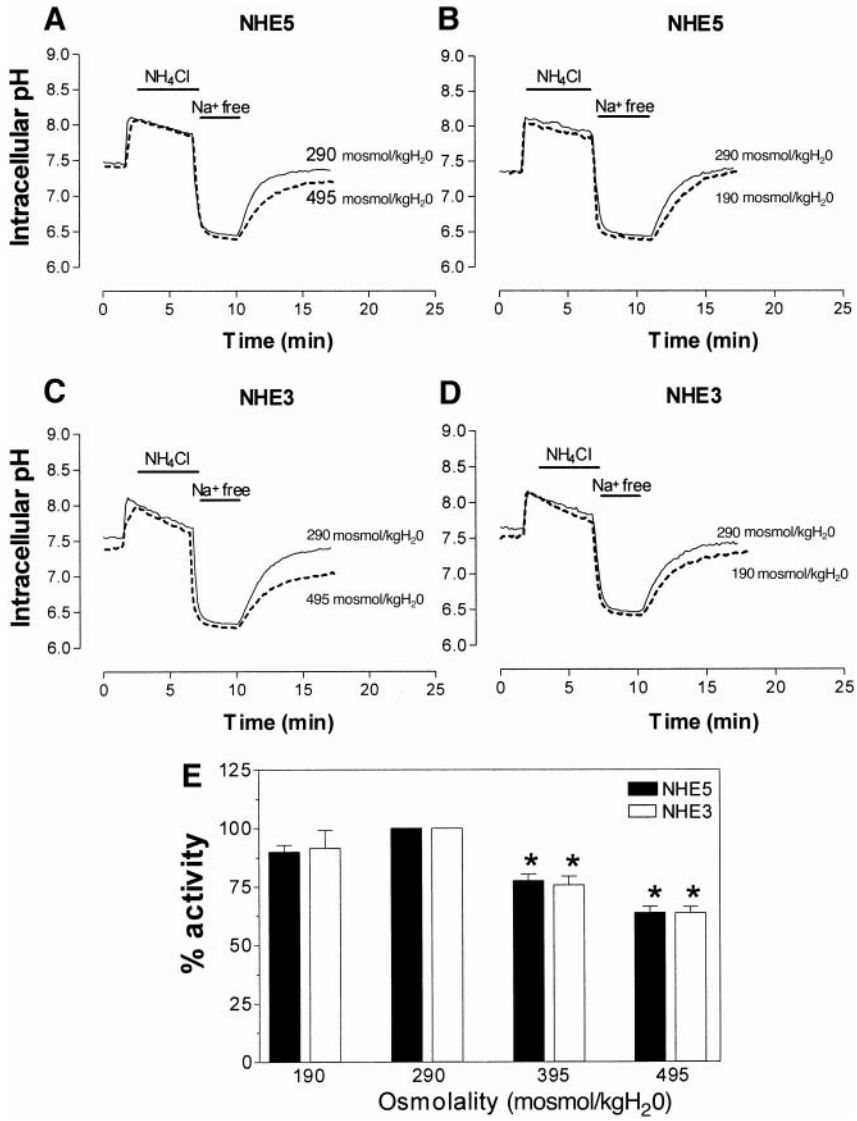

Fig. 5. Acute inhibition of rat NHE5 activity by changes in osmolality. PS120 fibroblast cells expressing either NHE5 or NHE3 were acid loaded using the NH_4^+ prepulse technique (as described in MATERIALS AND METHODS). *A* (NHE5) and *C* (NHE3) show that increasing the osmolality of the external solution from 290 to 495 significantly blunted the rate of pH recovery from an acid load. In contrast, exposure to hypotonic medium (190 mosmol/kgH_2O) had no significant effect on NHE5 or NHE3 (*B* and *D*, respectively). *E*: the effects of changing osmolality on NHE5 and NHE3. At 395 mosmol/kgH_2O, the pH recovery rates for NHE5 and NHE3 were inhibited by 22 and 25%, respectively. Increasing the osmolality to 495 mosmol/kgH_2O resulted in a further inhibition of the recovery rates of both NHE5 and NHE3 to 36%. Data shown represent the means ± SE of $n \geq 30$ cells from 3 different experiments for each condition. *Significant differences from control.

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over, NHE5 is likely an electroneutral exchanger, as previously shown for other Na^+/H^+ exchanger isoforms, since depolarization of the plasma membrane (external K^+ was raised from 5.73 to 50 mM while keeping the Na^+ concentration constant at 90 mM) did not significantly affect the rate of pH recovery (data not shown). This latter result also indicates that external K^+ does not significantly inhibit Na^+/H^+ exchange by competing with Na^+ for the same binding site as previously demonstrated for NHE1 (29).

Regulation of NHE5 by cell volume. In many cell types, including neuronal cells (1), the regulation of Na^+/H^+ exchanger activity can be associated with changes in cell volume. Osmotic shrinkage often results in activation of Na^+/H^+ exchange, which is generally coupled with Cl^-/HCO_3^- exchange. Shrinkage-induced activation of these exchangers thus results in a net intracellular accumulation of NaCl and an obligatory water influx to produce cell swelling, a response termed regulatory volume increase or RVI. However, Fig. 5*A* shows that increasing the tonicity of the external solution from ~290 to ~490 mosmol/kgH_2O significantly inhibited the rate of pH recovery by cells expressing NHE5 (~35% inhibition), suggesting that it is not involved in the RVI response. As previously reported (19), hyperosmotic shock inhibited NHE3 (Fig. 5*C*). The magnitude of this inhibitory response was reduced for both NHE5 and NHE3 when the osmotic shock was decreased to ~390 mosmol/kgH_2O (Fig. 5*E*). In contrast, cell swelling induced by diluting the external solution to 190 mosmol/kgH_2O had no significant effect on the transport rate of NHE5 (Fig. 5*B*) or NHE3 (Fig. 5*D*). The response of NHE5 to osmotic manipulations was thus qualitatively similar to those reported for rat NHE3 (Ref. 19 and as reproduced here), but quite different from those previously seen in rat NHE1 and NHE2 (19).

Regulation of rat NHE5 by PKC. PKC activation enhances the Na^+/H^+ exchanger activity of rat NHE1 and NHE2 but inhibits NHE3 (18). Figure 6*A* (and as summarized in Fig. 7*A*) shows that activation of PKC by the phorbol ester PMA inhibited the activity of NHE5 >70%. In contrast, the inactive phorbol ester, 4α-PMA, produced a relatively small decrease in the pH recovery rate of NHE5 that was not statistically significant (Figs. 6*B* and 7*A*). In agreement with previous studies in which rat NHE1 and NHE3 were expressed in AP-1 cells (18), PMA inhibited NHE3 >50% (Fig. 6*C*). In contrast, NHE1 activity was enhanced >25% (Fig. 6*D*, see also Ref. 19). Moreover, the PKC antagonist chelerythrine chloride blunted the PMA-induced inhibition of NHE5 activity (Fig. 7*B*), consistent with PMA acting on NHE5 through a PKC-dependent phosphorylation process.

Regulation of rat NHE5 by PKA. Na^+/H^+ exchanger activity is often modified by phosphorylation-dependent events, possibly including serine/threonine sites located in the cytoplasmic carboxy terminus of the transporter (21). A number of potential phosphorylation sites are in this "regulatory" domain of rat NHE5, including PKA-dependent sites (2). To examine the role of PKA in regulating Na^+/H^+ exchanger activity, PS120 cells expressing NHE5 were treated with agents known to activate this pathway.

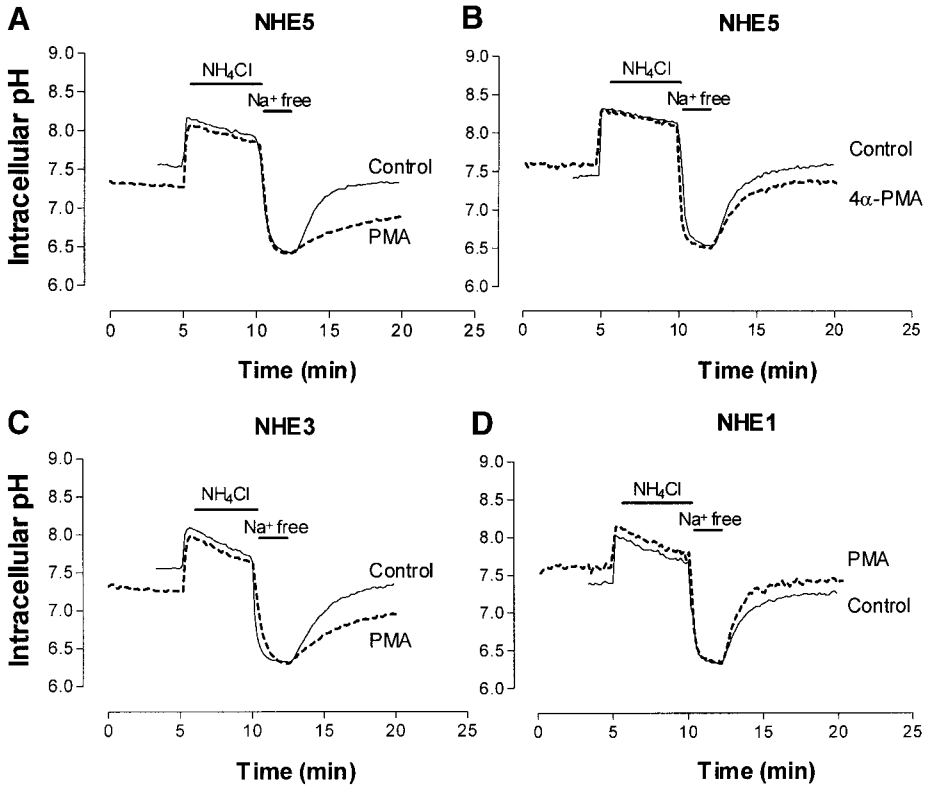

Fig. 6. Acute inhibition of rat NHE5 activity by the protein kinase C (PKC) agonist phorbol 12-myristate 13-acetate (PMA). Rat NHE5-expressing PS120 cells were incubated in serum-free media overnight before experimentation and then loaded with BCECF. To examine the regulation of NHE5 by PKC agonists, a paired NH_4^+ prepulse protocol was used in which cells were acid loaded, and, following recovery, acid loaded a second time in the presence of agonist. The untreated control (solid line) and experimental (dotted line) portions of the traces were overlaid to facilitate comparisons. After the initial control pulse, the same cells were incubated for 20 min before as well as during the experiment in medium containing the indicated agent: *A*, NHE5 treated with 1 μM PMA; *B*, NHE5 treated with 1 μM 4α-PMA; *C*, NHE3 treated with 1 μM PMA; and *D*, NHE1 treated with 1 μM PMA. Traces are the average results from $n \geq 30$ cells from 3 different experiments.

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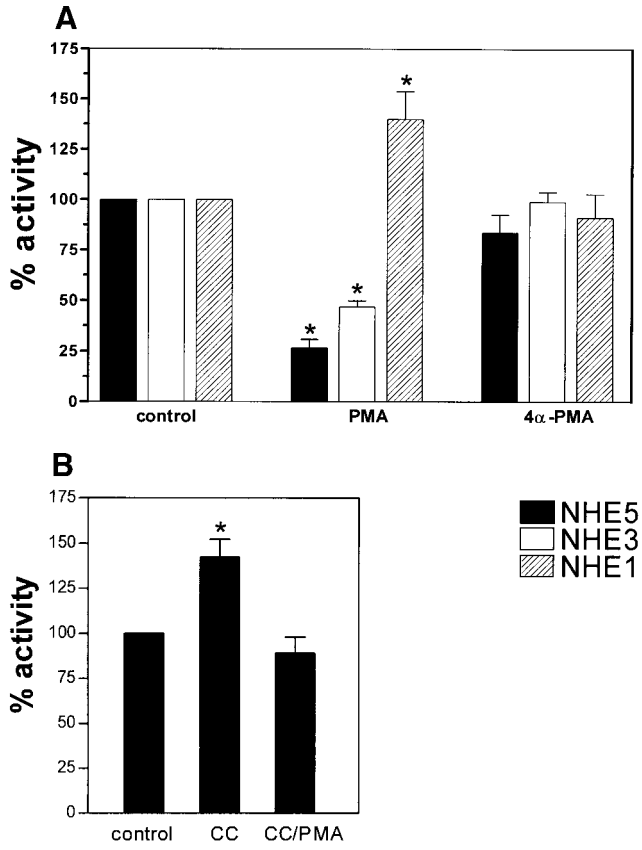

Fig. 7. Effects of PKC agonists and inhibitors on rat NHE5, NHE3, and NHE1 activity. PS120 cells were incubated in serum-free media overnight before experimentation and then loaded with BCECF. *A*: effects of PMA and its inactive analog 4α-PMA on the rate of Na^+-dependent acid recovery in cells expressing NHE5, NHE3, and NHE1. Cells were treated with either 1 μM PMA or 1 μM 4α-PMA for 20 min before acid loading and then throughout the acid-loading and recovery periods. PMA inhibited recovery of NHE5 and NHE3 by 73 and 53%, respectively. In contrast, PMA treatment activated NHE1, increasing its recovery rate by 40%. The inactive phorbol ester, 4α-PMA (1 μM), did not significantly effect the recovery rate of any of the NHE isoforms. *B*: effects of 1 μM chelerythrine chloride (CC) on NHE5. Simultaneous treatment with CC alone resulted in a significant 42% increase in the recovery rate. Treatment with CC prevented the PMA-induced inhibition of NHE5. Data shown represent the means ± SE of $n \geq 30$ cells from 3 different experiments for each condition. *Significant differences from control.

As shown in Fig. 8*A* and as summarized in Fig. 9, NHE5 activity was inhibited >60% by acute exposure to 10 μM forskolin, an agent that increases intracellular cAMP by activating adenylate cyclase. In contrast, there was no significant effect of the inactive forskolin analog 1,9-DDF on NHE5 activity (Fig. 8*B*). To verify that the effect of forskolin was mediated through cAMP, we examined the response of NHE5 to the cell-permeant cAMP analog 8-Br-cAMP. Like forskolin, 8-Br-cAMP (100 μM) inhibited NHE5 activity, nearly 70% (Fig. 8*C*), and the potent PKA antagonist H-89 reversed the effects of forskolin (Fig. 9*B*), suggesting that inhibition of NHE5 by cAMP involves a PKA-dependent process. Moreover, the PKC inhibitor chelerythrine chloride had no effect on the forskolin-induced inhibition of NHE5 (Fig. 9, compare *B* with *A*), indicating that this cAMP-dependent response did not involve the PKC signaling pathway. In contrast to cAMP, 8-Br-cGMP produced only a subtle change in NHE5 exchanger activity that was not statistically significant (Fig. 8*D*), suggesting that cGMP-dependent kinases do not regulate NHE5. Together, these results demonstrate that PKA activation inhibits NHE5 activity. As observed for NHE5, Fig. 9*A* shows that NHE3 was inhibited by forskolin and 8-Br-cAMP, whereas these agents did not have a significant effect on NHE1 activity.

A dramatic inhibition of both rat NHE3 and NHE5 occurred through a PKA-dependent process when expressed in PS120 cells (Figs. 8 and 9). PKA-dependent phosphorylation (inhibition) of rabbit NHE3 is thought to require association of NHE3 with PKA through "linker" proteins (46); however, PS120 cells apparently lack the PKA-binding protein ezrin and the adaptor protein NHERF. Therefore, regulation of rabbit NHE3 by PKA in this cell type requires coexpression of ezrin and NHERF (50). However, we did not coexpress ezrin and NHERF. The simplest explanation for this discrepancy may be differences in the proteins expressed by the PS120 clonal isolates used in our study and those in previous studies. To test this possibility, RNA was isolated from PS120 cells, and RT-PCR was performed using probes designed to amplify NHERF and ezrin. Figure 10 demonstrates that transcripts for both NHERF (*A*) and ezrin (*B*) were detected in all tissues and cell lines tested, including the hamster kidney (*lane* 3), the hamster-derived cell line CCL-39 (*lane* 4), PS120 cells (*lane* 5; derived from CCL-39 cells), PS120/NHE3 cells (*lane* 6), and PS120/NHE5 cells (*lane* 7).

DISCUSSION

We have a general understanding of the properties of Na^+/H^+ exchange activity in the central nervous system (5, 10, 16, 25, 26, 34, 41, 48); however, relatively little is known about the expression and function of specific NHE isoforms. NHE1, the housekeeping isoform thought to be the primary regulator of pH_i in most cells, is expressed throughout the brain, and its expression is critical for normal postnatal development of the mouse central nervous system. Approximately 2 wk after birth, mice lacking expression of functional NHE1 display growth retardation, severe ataxia, and epileptic-like seizures associated with increased mortality (4, 15). In contrast, the epithelial isoform NHE3 is not present in detectable levels in the brain, with the exception of the cerebellum (9), and knocking out this gene failed to create demonstrable neurological consequences (38). Two other epithelial isoforms, NHE2 and NHE4, are expressed in the brain, although apparently to a lesser extent than NHE1 (32, 45). Targeted disruption of NHE2 failed to produce obvious neurological defects (37). More recently, a fifth plasma membrane isoform NHE5 was cloned (2, 3). NHE5 is expressed throughout the brain (3), suggesting that it may serve a specialized function in neurons. Initial characterization of the kinetic properties of human NHE5 suggests

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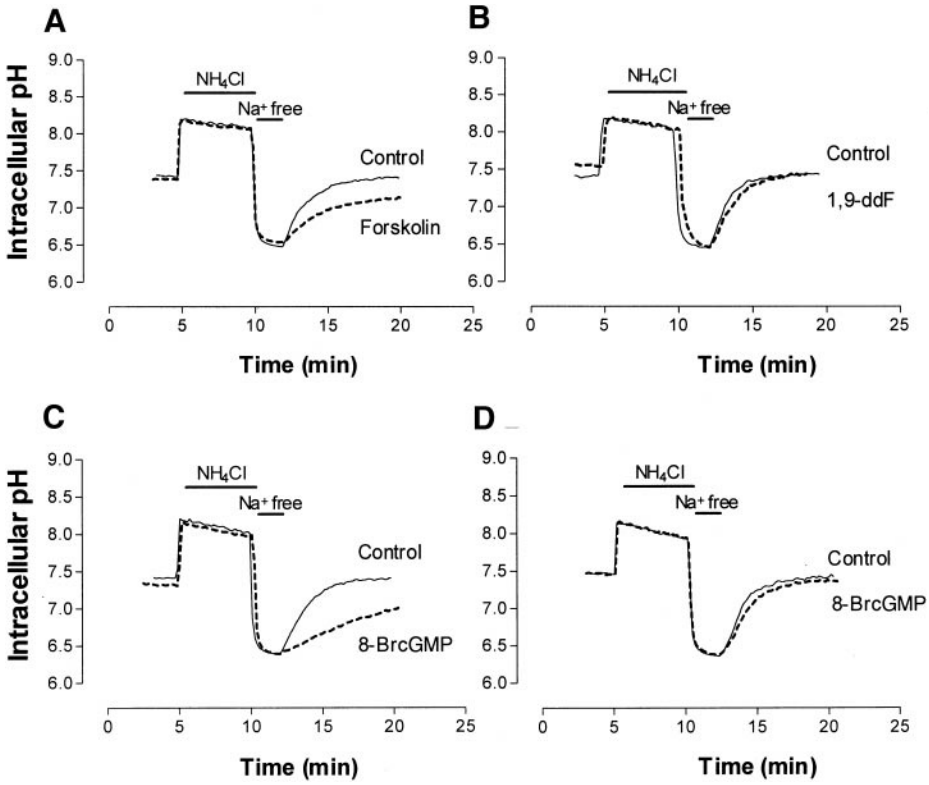

Fig. 8. Acute inhibition of rat NHE5 activity by cAMP agonists. Rat NHE5-expressing PS120 cells were incubated in serum-free media overnight before experimentation and then loaded with BCECF. To examine the regulation of NHE5 by cAMP agonists, a paired NH_4^+ prepulse protocol was used in which the same cells were acid loaded, and then following recovery, acid loaded a second time in the presence of agonist. The untreated control (solid lines) and experimental (dotted lines) portions of the traces were overlaid to facilitate comparisons. Immediately following the control pulse, the same cells were incubated for 20 min before as well as during the experiment in medium containing one of the following: *A*, 10 μM forskolin; *B*, 10 μM 1,9-dideoxyforskolin (1,9-DDF); *C*, 100 μM 8-bromo-cAMP (8-Br-cAMP); or *D*, 100 μM 8-bromo-cGMP (8-Br-cGMP). Each trace is the average result from $n \geq 30$ cells from 3 different preparations.

that it behaves much like the amiloride-resistant Na^+/H^+ exchanger activity present in hippocampal neurons (39).

To gain a better understanding of NHE5 function and its regulation, we stably expressed this protein in cells lacking Na^+/H^+ exchange. Our results indicate that many of the basic functional properties (e.g., EIPA sensitivity and ion selectivity) of rat NHE5 under physiological conditions (i.e., in a high external Na^+ concentration) are similar to human NHE5 (39). The amino acid sequence of rat NHE5 (2) and its sensitivity to the Na^+/H^+ exchange inhibitor EIPA are most like NHE3 (Fig. 1, see also Ref. 29). These results likely reflect the close similarity of the NHE5 and NHE3 ion-transporting domain (62% identity) that also contains the predicted amiloride-binding region (44). Like other members of the NHE family (with the exception of NHE4, see Ref. 13), K^+ and Rb^+ were nontransportable by NHE5. The affinity of NHE5 for Na^+ is relatively low ($K_{Na} \approx$ 27 mM), being intermediate to NHE1 and NHE3 (29), 10 and 5 mM, respectively, and NHE2 at 50 mM Na^+ (49). The Na^+ affinity reported for the Na^+/H^+ exchange present in rat hippocampal neurons ($K_{Na} \approx$ 23–26 mM, see Ref. 34) is comparable to rat as well as human NHE5 ($K_{Na} \approx$ 19 mM, see Ref. 39), consistent with NHE5 being expressed in this region of the brain (2). The transport activity of rat NHE5 as a function of the intracellular H^+ concentration was similar to that reported for other NHE isoforms (23, 29). The Hill coefficient of rat NHE5 was ~3 for the intracellular proton concentration with a half-maximal activity near pH 6.9. These results are different, however, from those reported for human NHE5 in which an apparent first-order dependence on the intracellular proton concentration was observed with a half-maximal activity at pH ~6.43 (39). The Na^+/H^+ exchange in rat hippocampal neurons also apparently displays first-order dependence on the intracellular H^+ concentration, but with a half-maximal activity greater than pH 6.8, closer to the results of the present study (34).

In sharp contrast to Na^+/H^+ exchange in rat hippocampal neurons (34) in which the pH_i recovery rate from an acid load in physiological external Na^+ was comparable to that in the presence of Li^+, NHE5 exchanged Li^+ at a very low rate compared with Na^+ (Fig. 4). Other members of the NHE gene family also transport Li^+ at a slower rate than Na^+, although the differences in rates of translocation for Na^+ and Li^+ are not as dramatic (see Refs. 17 and 29, and as confirmed in the present study). This observation may relate to differences in the affinity of the Na^+-binding site for Li^+; that is, Li^+ competition with Na^+ for this site is an order of magnitude stronger in NHE5 (39) than in other NHE isoforms (29), resulting in a slower transport rate.

Together, it appears that the Na^+/H^+ exchanger activity described in rat hippocampal neurons cannot be easily attributed to any single NHE isoform (34). The complete insensitivity of this exchanger to amiloride (1 mM) and its derivative *N*,*N*-hexamethyleneamiloride (100 μM) contrast with the inhibition constants of these reagents and related compounds for NHE5 (2, 39). Moreover, the comparable transport rate for Na^+ and Li^+ on the Na^+/H^+ exchanger in the hippocampus

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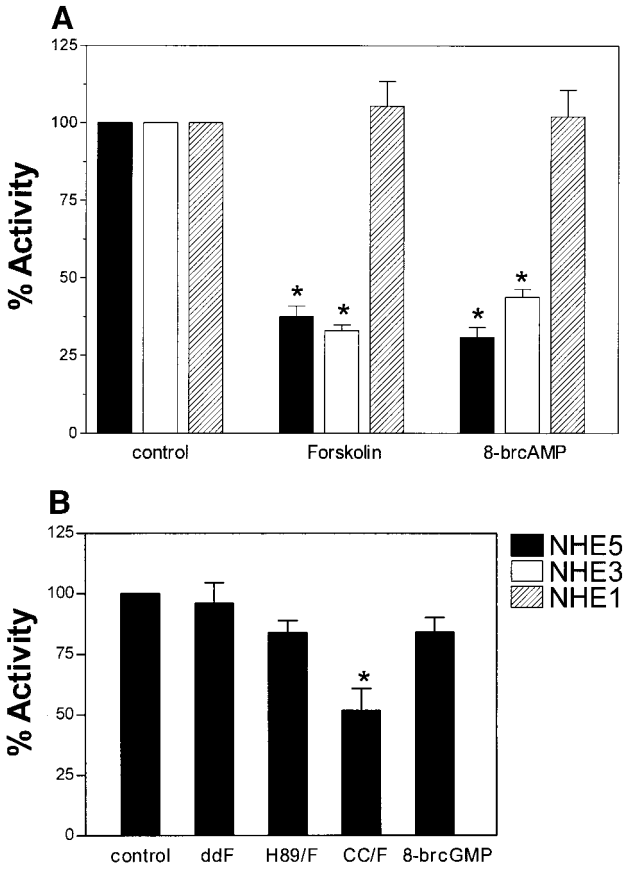

Fig. 9. Effects of protein kinase A (PKA) agonists and inhibitors on rat NHE5, NHE3, and NHE1 activity. PS120 cells were incubated in serum-free media overnight before experimentation and then loaded with BCECF (control = untreated or treated with inhibitor only). *A*: effects of forskolin and 8-Br-cAMP on the rate of Na^+-dependent acid recovery in cells expressing NHE5, NHE3, or NHE1. Cells were treated with 10 μM forskolin or 100 μM 8-Br-cAMP for 20 min and throughout the acid-loading and recovery periods. *B*: cells expressing rat NHE5 were treated as in *A* but with either 10 μM 1,9-DDF, 10 μM forskolin (F) in the presence of either 100 μM H-89 (a PKA inhibitor), or 1 μM CC (a PKC inhibitor). H-89 blocked ~75% of the forskolin-induced inhibition of acid recovery of NHE5, whereas CC was not effective. In addition, treatment with 100 μM 8-Br-cGMP, a cGMP analog, resulted in no significant effect on activity. The rate of Na^+-dependent acid recovery was calculated from the slope of the initial linear portion of experimental and control regions for each cell. Data represent the means ± SE of $n \geq 30$ cells from 3 different experiments for each condition. *Significant differences from control.

is inconsistent with NHE5 expression (present study and Ref. 39). We cannot rule out the possibility that when expressed in neurons, the inhibitor sensitivity and ion selectivity of NHE5 are dramatically altered; however, this seems unlikely.

The cytoplasmic carboxy termini of NHE proteins contain elements thought to be the primary sites for regulation of transport activity, including sites for phosphorylation by serine/threonine kinases (18). The carboxy-terminal region contains the largest degree of divergence within the NHE family of proteins. This property holds true for NHE5 as well; i.e., NHE5 has <20% identity with NHE1, NHE2, or NHE4, and 26% identity with NHE3 in the carboxy termini of these proteins (2, 3). Therefore, it might be expected that the regulation of NHE5 activity by phosphorylation would be distinct, as shown for the other NHE isoforms (18, 23). Nevertheless, NHE5 responded to stimulation in a qualitatively similar manner to NHE3. NHE5 and NHE3 were inhibited to a comparable extent by PKA or PKC activation and by hypertonic shock. In contrast, NHE1 activity was not significantly affected by PKA activation but was enhanced by PKC stimulation (in the present studies) and when exposed to a hypertonic solution (see Ref. 19). Thus the regulation of NHE5 is qualitatively most like NHE3, the NHE isoform with which NHE5 shares the highest amino acid sequence identity.

The above regulation of NHE5, NHE3, and NHE1 is most likely associated with the carboxy terminus (18), possibly due to direct phosphorylation. Potential phosphorylation sites on NHE5 for PKC are located at Ser-593 and Ser-652, and PKA (and Ca^{2+}/calmodulin-dependent kinase II) sites are predicted at Ser-649, Ser-732, Ser-855, and Ser-857 (2). Of these PKA phosphorylation sites, only the homologous site in NHE5 for Ser-649 is present in rat NHE3 Ser-661 (32). However, a mutation at this site failed to have an effect on the sensitivity of NHE3 to forskolin (21). In contrast, mutation of Ser-605 prevented phosphorylation of rat NHE3 and blunted the inhibition induced by forskolin (21). This site aligns with the PKC site located at Ser-593 in rat NHE5. It is important to note that PKA-dependent inhibition (phosphorylation) of rabbit NHE3 is thought to require the physical association of

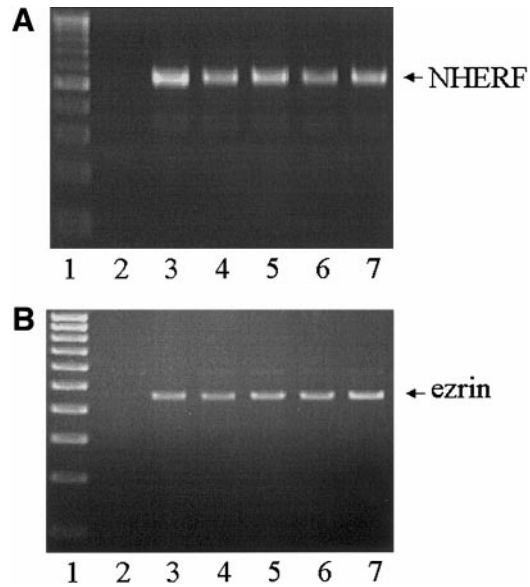

Fig. 10. PS120 fibroblast cells express Na^+/H^+ exchanger regulatory factor (NHERF) and ezrin. RT-PCR was used to test for NHERF (*A*) and ezrin (*B*) messages in hamster kidney mRNA (*lane* 3) and mRNA prepared from the Chinese hamster lung fibroblast cell line CCL-39 (*lane 4*), the sodium-proton exchanger-deficient cell line PS120 (*lane 5*), which was derived from CCL-39 cells, and the PS120/NHE3 (*lane 6*) and PS120/NHE5 (*lane 7*) lines, which were derived by stable transfection with the NHE3 and NHE5 isoforms, respectively. *Lane 1* contained mixed 100- and 500-bp ladders, and *lane 2* was a negative control lacking cDNA template. The identities of the NHERF and ezrin PCR products were verified by DNA sequencing.

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NHE3 with PKA through linker proteins (46). The PS120 cells used in previous studies apparently lacked the PKA-binding protein ezrin and the adaptor protein NHERF, thus regulation of rabbit NHE3 by PKA in this cell type requires coexpression of these proteins (50). Nevertheless, we clearly observed a dramatic inhibition of both rat NHE3 and NHE5 through a PKA-dependent process when expressed in PS120 cells. Although we did not rule out the possibility that fundamentally different mechanisms may be involved in the PKA-induced inhibition of the rat NHE3 protein, the basis for this apparent discrepancy appears to reflect differences in the PS120 clonal isolates used in our study. Figure 10 demonstrates that transcripts for both NHERF and ezrin are expressed in PS120 cells. Regardless of the mechanism(s) involved, future studies should identify the molecular machinery that regulates rat NHE5 via PKA and PKC activation and allow us to ascribe in situ function(s).

NHE5 was also regulated by changes in the cell volume in the present studies. Cell shrinkage inhibited activity, whereas swelling had no detectable effect. These results are most like those observed for NHE3, where a hypertonic solution blunted activity, and swelling was without effect (19). Conversely, NHE1 and NHE2 are activated by cell shrinkage and inhibited by swelling (19). NHE4 is coexpressed in some of the same regions of the brain (7) as NHE5 (2, 3). However, unlike NHE5, NHE4 is apparently activated by hyperosmolar conditions (8), suggesting possible overlapping, yet antagonistic, functions for these two exchangers.

In summary, we have characterized some of the kinetic and regulatory properties of rat NHE5. It is interesting to note that brain-specific NHE5 behaves qualitatively much like NHE3, an exchanger with high expression within specific regions of renal and intestinal epithelia (32). In both of these tissues, the apical membrane NHE3 plays a major role in Na^+ absorption, as demonstrated in mice lacking expression of this protein (11, 38). Clearly, the function of NHE5 is quite different in neurons, where NHE5 may complement the pH housekeeping exchanger NHE1. This redundancy may be necessary in some neurons in which high metabolic activity under physiological and pathophysiological conditions results in substantial acid production. However, the regulation of NHE1 is quite different from NHE5. In contrast to NHE1 (18, 19, 23), kinase activation and cell shrinkage inhibit NHE5 activity, suggesting that these exchangers may be most active under different (patho)physiological conditions or possibly have antagonistic actions on neuronal activity. Moreover, the p*K* of rat NHE5 is near pH 6.9, indicating that this exchanger is active at resting pH and is poised to respond when the cell is acid loaded. At present, we can only speculate the functional significance of NHE5 activity in the brain and the response of this exchanger to the activation of protein kinases A and C and cell shrinkage. The spontaneous and epileptiform bioelectric activity of hippocampal CA3 neurons is suppressed by intracellular acidification (6). This acidification has been shown to provide protective advantage during an ischemic challenge (22). Therefore, protein kinase-dependent inhibition of NHE5 might yield protection from postischemic brain damage. Nevertheless, it is clear that Na^+/H^+ exchange activity is required for normal electrical activity, at least for NHE1-mediated exchanger activity. Null mutations of this latter Na^+/H^+ exchanger gene produce epileptic seizures (4, 15). Because of the complexity of the brain, targeted disruption of the *Nhe5* gene will likely be necessary to sort out its functions.

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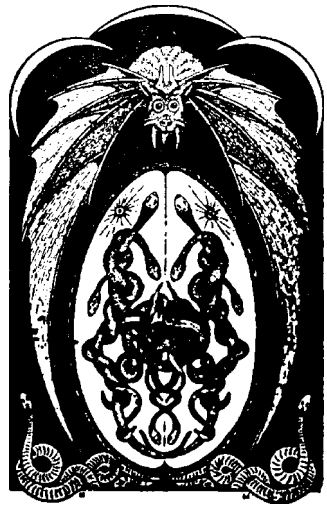

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