J. Membrane Biol. 160, 183–192 (1997)

The Journal of
Membrane
Biology

Inverse Relationship Between Membrane Lipid Fluidity and Activity of Na^+/H^+ Exchangers, NHE1 and NHE3, in Transfected Fibroblasts

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Received: 23 January 1997/Revised: 1 August 1997

Abstract. This report presents a study of the effects of the membrane fluidizer, benzyl alcohol, on NHE isoforms 1 and 3. Using transfectants of an NHE-deficient fibroblast, we analyzed each isoform separately. An increase in membrane fluidity resulted in a decrease of ≈50% in the specific activities of both NHE1 and NHE3. Only V_{max} was affected; K_{Na} was unchanged. This effect was specific, as Na^+, K^+, ATPase activity was slightly stimulated. Inhibition of NHE1 and NHE3 was reversible and *de novo* protein synthesis was not required to restore NHE activity after washout of fluidizer. Inhibition kinetics of NHE1 by amiloride, 5-(N,N-dimethyl)amiloride (DMA), 5-(N-hexamethyl)amiloride (HMA) and 5-(N-ethyl-N-isopropyl)amiloride (EIPA) were largely unchanged. Half-maximal inhibition of NHE3 was also reached at approximately the same concentrations of amiloride and analogues in control and benzyl alcohol treated, suggesting that the amiloride binding site was unaffected. Inhibition of vesicular transport by incubation at 4°C augmented the benzyl alcohol inhibition of NHE activity, suggesting that the fluidizer effect does not solely involve vesicle trafficking. In summary, our data demonstrate that the physical state of membrane lipids (fluidity) influences Na^+/H^+ exchange and may represent a physiological regulatory mechanism of NHE1 and NHE3 activity.

Key words: Ion transport — Na^+/H^+ exchange — NHE — Membrane fluidity — Benzyl alcohol

Introduction

Differences in lipid composition, the physical state (fluidity) of the plasma membrane and protein-lipid interactions have been shown to significantly affect the activities of a number of intestinal epithelial membrane proteins involved in nutrient or ion transport (Dudeja et al., 1989). Previous studies (Brasitus et al., 1986; Dudeja, Foster & Brasitus, 1987) have described the stimulatory effects of membrane fluidizing agents on Na^+/H^+ exchange activity in rat intestinal brush border membrane vesicles, a process that mediates a major component of transcellular sodium absorption by the gut. The activity of plasma membrane proteins, like NHEs, can be affected in many ways, including phosphorylation of regulatory domains and by association with regulatory proteins. However, another, albeit less well understood, regulatory process may involve transient changes in local plasma membrane lipid composition or global and long term perturbations caused by disease states and characterized by altered lipid fluidity.

Analyses of how membrane lipids and altered fluidity might affect Na^+/H^+ exchangers' function is confounded by the fact that intestinal epithelial cells appear to express multiple isoforms. Two isoforms, NHE2 (Hoogerwerf et al., 1996) and NHE3 (Biemesderfer et al., 1993; Bookstein et al., 1993) are known to localize to brush borders, while the ubiquitously expressed NHE1 appears to be restricted to the basolateral membrane domain of these polarized cells (Tse et al., 1991). Most cultured cell systems which naturally express a brush border NHE also express the housekeeping NHE1 isoform. Therefore, to analyze the interaction between lipid environment and a single exchanger in identical environments, we have utilized an NHE-deficient fibroblast cell line (PS120) (Pouyssegur et al., 1984). Thus we can analyze the effects of altered membrane fluidity on the activity of NHE1 or NHE3, without the confounding background of other endogenously expressed NHEs.

We report here that, in contrast to the stimulation of Na^+/H^+ exchange activity observed in intestinal brush

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border membrane vesicles, the activities of NHE1 and NHE3 in transfected fibroblasts are inhibited by benzyl alcohol. This effect appears to selectively affect NHEs, as increased membrane fluidity stimulated Na^+, K^+-ATPase activity. Thus, perturbations of the membrane lipid milieu may play a role in modifying Na transport processes which are mediated by Na^+/H^+ exchange.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium, penicillin, streptomycin, trypsin-EDTA and fetal bovine serum were obtained from Life Technologies, (Gibco/BRL). Cell culture plates and flasks were purchased from Corning or Costar. [^{22}Na]Cl (1,000 Ci/g) was from DuPont NEN. Amiloride and analogues (5-(N-ethyl-N-Isopropyl)amiloride (EIPA), and 5-(N-hexamethyl)amiloride (HMA) were from Molecular Probes (5-N,N-dimethyl)amiloride (DMA) was a gift from T. Kleyman, University of Pennsylvania. Other reagents were purchased from Sigma-Aldrich or Fisher Scientific.

Cells

The NHE-deficient fibroblast cell line (PS120) (Pouyssegur et al., 1984) was provided by J. Pouyssegur (Université de Nice). These cells were lipofectin (Life Technologies) transfected with full length cDNA for rat NHE1 and NHE3 on the pCB6+ vector (gift of J.M. Stinski, University of Iowa). These cDNAs (Orlowski, Kandasamy & Shull, 1992) were gifts from G. Shull (University of Cincinnati) and J. Orlowski (McGill University). Each G418-resistant and acid-tolerant clonal transfectant was verified by Northern and Western blot analyses using isoform specific probes and antibodies. The clones, designated here as PS/NHE1 and PS/NHE3, were periodically reselected by survival to acid load, as described previously (Bookstein et al., 1993).

Measurement of Plasma Membrane Lipid Fluidity

Steady-state fluorescence polarization was measured on the SLM 4800C fluorescence spectrofluorometer (SLM-Aminco, Urbana, IL) utilizing the lipid-soluble fluorophore trimethyl ammonium 1,6-diphenyl-1,3,5-hexatriene (TMA.DPH). This probe, a cationic derivative of DPH, has been used successfully to assess the plasma membrane lipid fluidity of a variety of intact cells (Prendergast, Haugland & Callahan, 1981; Straume & Litman, 1988). The positive charge in the molecule provides a plasma membrane surface anchor and, therefore, improves its specific localization in the plasma membranes. The methods used to load the cells with this probe and quantification of the fluorescence have been described in detail (Brasitus & Dudeja, 1985; Dudeja et al., 1995). Briefly, a 0.5 mM stock of TMA.DPH in dimethyl formamide is diluted to a final concentration of 1 μM in the PBS buffer (pH 7.5) containing cells. After proper mixing for 1–3 min, the steady-state fluorescence polarization was immediately assessed (prolonged incubation causes the internalization of the probe) with excitation at 340 nm and emission at 425 nm as previously described (Straume & Litman, 1988). Corrections for light scattering (cell suspension minus probe) were performed routinely. Moreover, no change in the excited state-lifetime, as assessed by total fluorescence intensity, was demonstrated for the probe in the presence or absence of benzyl alcohol in each preparation examined.

Kinetic Analysis of Sodium Uptake

[$^{22}Na^+$] fluxes were measured as described previously (Bookstein et al., 1994). Cells were grown to confluence in 24 well dishes and were given fresh media with 10% serum 16 to 18 hr prior to the experiment. The Na/H exchanger was activated by incubation for 60 min., at 37°C, in isotonic NH_4Cl prepulse buffer (in mM): 50 NH_4Cl, 70 choline chloride, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 glucose, 15 HEPES, pH 7.0 and washing with isotonic buffered choline-Cl. Flux buffer consisted of (in mM): 102.5 choline Cl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 2 glucose, 1 ouabain, 15 HEPES pH 7.4, 20 NaCl with 1 mCi/ml [^{22}Na]Cl. Uptake was determined to be linear for 8 min (*data not shown*). Influx was stopped after 4 min by four washes in cold phosphate buffered saline with 1 mM $MgCl_2$ and 2 mM $CaCl_2$. Except where noted, uptake was measured in the absence and presence of 500 μM DMA. Following the 4-min flux, the cells were washed and extracted in 0.5% (w/v) SDS. An aliquot was removed to count intracellular [$^{22}Na^+$] in a liquid scintillation counter and protein was measured by the bicinchoninic acid procedure. To calculate the nmoles of Na^+ taken up, the average dpm of the samples was divided by the specific activity of the [$^{22}Na^+$] used at each Na^+ concentration. Fluxes are expressed as the DMA-inhibitable component of total flux (unless stated otherwise), in nmoles of Na^+ taken up by the cells per minute per mg of total protein.

Measurement of Internal pH

After determination of cell water space with [^{3}H] H_2O and correction for radioactivity trapped in extracellular space (using D-[^{3}H]mannitol), as described in L'Allemain, et al. (L'Allemain, Paris & Pouyssegur, 1984), pH_i was calculated by the distribution of [^{14}C]benzoic acid, according to the equation $pH_i = pH_o + \log\beta_i/\beta_o$, where β is the concentration of benzoic acid (in dpm/ul). All radioactive chemicals were from New England Nuclear.

Enzymatic Assay of Na^+, K^+, ATPase

Essentially according to Garrahan, Pouchan & Rega (1969). Cells were acidified as described above and incubated in fresh complete media, plus or minus 10 mM benzyl alcohol, for 4 min. Membranes were prepared by scraping cells into PBS, pelleting and homogenizing in lysis buffer (in mM): 10 Tris pH 7.4, 2 EGTA, 2 EDTA, 1 PMSF, with 10 μg/ml leupeptin and aprotinin by 20 strokes of a glass pestle homogenizer. The homogenate was centrifuged at 10,000 × *g* for 10 min, at 4°C to remove unbroken cells, nuclei, and mitochondria, and the supernatant was spun 30 min at 4°C to pellet the membrane fraction. Membranes were resuspended in homogenization buffer and protein measured using the bicinchoninic acid procedure. One hundred micrograms were used to measure Na^+, K^+, ATPase activity by the K-stimulated phosphatase assay of Garrahan et al. (1969). The difference of phosphatase activity in the presence and absence of K^+ was defined as the K-stimulated activity.

Results

Dose-dependent Effects of Membrane Fluidizer on NHE Activity

Previous studies on the relationship between membrane fluidity and NHE activity had been conducted in intes-

Table 1. Effect of benzyl alcohol on fibroblast membrane lipid fluidity

BenzylOH [mM]	r′ at 25°C	% change
0	0.278 ± 0.003	0
5	0.274 ± 0.003	1.4
10	0.273 ± 0.004	3.5
20	0.268 ± 0.004	3.6
30	0.266 ± 0.004	4.3
40	0.260 ± 0.005	6.5
50	0.258 ± 0.005	7.2

Fluorescence polarization studies, utilizing TMA.DPH as fluorescent probe, were done on the PS120 fibroblasts to determine dose-response relationship of benzyl alcohol on plasma membrane lipid fluidity. Values represent mean ± SEM of 3–4 independent observations of separate cell preparations (each value being an average of ~10 determinations).

tinal brush border membrane vesicles, disrupted cells which contain multiple NHE isoforms. To understand the interaction between lipid environment and a single exchanger isoform, we transfected the well-characterized NHE-deficient mutant fibroblast cell line, PS120 (Pouyssegur et al., 1984) with NHE1 or NHE3 cDNAs (both rat) as described in Materials and Methods. First, we determined the relative fluidity increases of the fibroblast plasma membranes at various concentrations of a commonly used fluidizer-benzyl alcohol. As shown in Table 1, at concentrations of 10, 20 and 30 mM, benzyl alcohol increased membrane fluidity by 3.5, 3.6, and 4.3%, respectively. Next, we measured NHE activity in the presence of increasing concentrations of benzyl alcohol (Fig. 1). With 10-mM benzyl alcohol in the flux solution (Fig. 1*A*), NHE1 activity was significantly decreased as compared to 0–3-mM fluidizer concentrations. In the presence of 30-mM benzyl alcohol, NHE1 activity was 16 ± 7% of the DMA-inhibitable flux in the absence of fluidizer. NHE3 (Fig. 1*B*) showed similar responsiveness to this fluidizer. At 10-mM benzyl alcohol, activity was reduced to a statistically significant 68 ± 7% of control ($P < 0.1$). At 30 mM, activity was reduced to 22 ± 10% of the activity observed in the absence of benzyl alcohol.

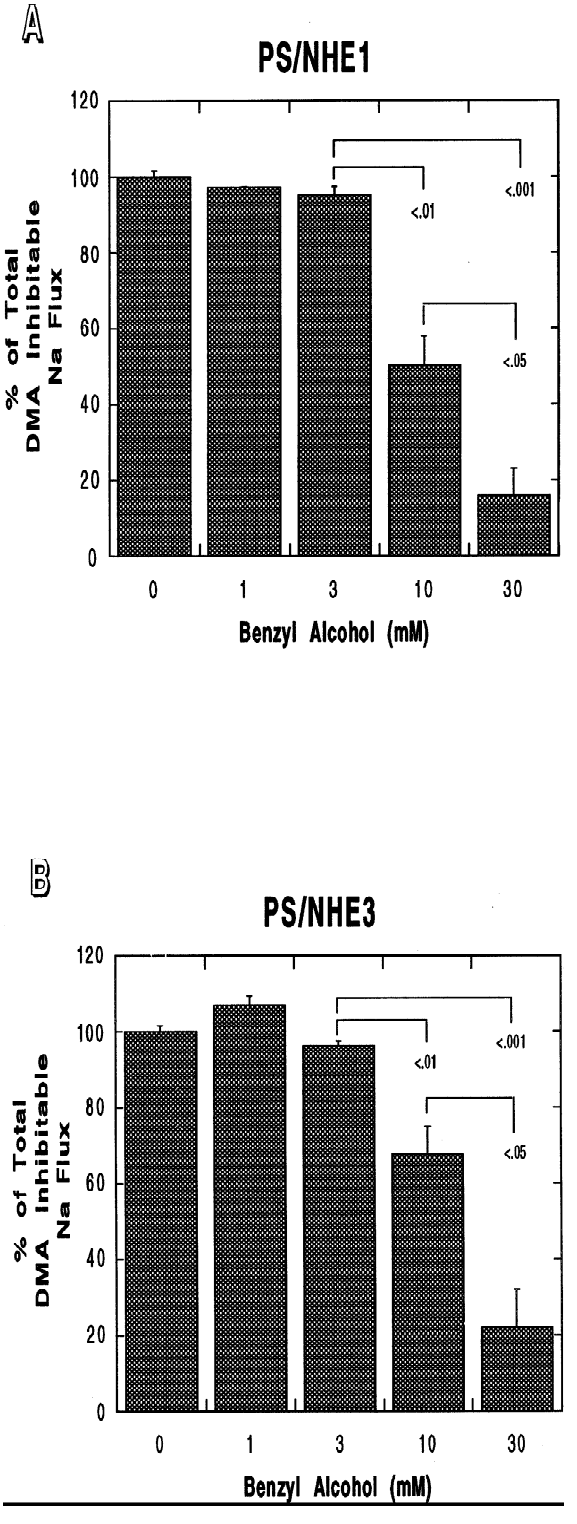

Fig. 1. Dose-response inhibition of NHE activity by benzyl alcohol. Specific activities (Na influx) of NHE1 and NHE3 were determined in the presence of increasing concentrations of the fluidizer, benzyl alcohol. The cells were acid loaded prior to assay as described in Materials and Methods. Flux activity was calculated by measuring the amount of [^{22}Na] taken up by the cells during a 4-min flux, when uptake was still linear (*data not shown*) and correcting for cold $[Na]_o$ in the buffer. Extracellular Na concentration was 5 mM. Specific activity is defined as nM of Na taken up per minute per mg of protein. Specific activity in the absence of fluidizer (for each isoform) was arbitrarily set at 100%. Each succeeding point is presented as a percentage of that value, as indicated on the Y-axes. Shown on the X-axes are the concentrations (mM) of benzyl alcohol, present *only* during the 4-min flux period. All assays were done on rat NHE clonal, stable transfectants of the NHE-deficient PS120 Chinese Hamster fibroblasts. Shown in panel *A* is the dose response of NHE1 to benzyl alcohol. In panel *B*, the effects of benzyl alcohol on NHE3 activity is shown. Values represent an $n = 6$, mean ± SEM. *P* values are as shown; the experimental conditions compared for statistical significance (ANOVA) are bracketed.

Table 2. Effect of benzyl alcohol on pH_i

	PS/NHE-1	PS/NHE-3
Extracellular space (μl)	0.129 ± 0.024	0.132 ± 0.009
Cell H_2O (μl)	0.543 ± 0.104	0.617 ± 0.127
	pH_i	pH_i
Pre-acidification	6.92 ± 0.04	6.81 ± 0.10
Post-acidification	5.98 ± 0.15	6.01 ± 0.24
4 min Post-acidification DMA only	6.12 ± 0.06	6.04 ± 0.06
4 min Post-acidification DMA plus BenzylOH	6.11 ± 0.01	5.92 ± 0.11
4 min Post-acidification no additions	6.39 ± 0	6.31 ± 0.14

Confluent cells were acidified as described. Cell water space was measured with [^{3}H] H_2O to correct for radioactivity from D-[^{3}H] mannitol trapped in the extracellular space. Internal pH was determined prior to treatment with NH_4Cl saline and immediately after. Then pH_i was measured after a 4-min incubation with Na saline alone, Na saline with 500 μm DMA, or Na saline with 10-mM benzyl alcohol. The assay buffer also contained 500-μM DMA so that while flux was measured no further changes in pH_i occurred. $n = 4$. Mean ± SEM.

All subsequent experiments were performed in the presence of 10-mM benzyl alcohol, the lowest concentration which produced significant perturbation in NHE activities.

Incubation in 10-mM Benzyl Alcohol Does Not Alter pH_i

Because a relatively small alteration in membrane fluidity exerted such a significant effect on the activities of these NHE isoforms, we examined the possibility that benzyl alcohol or amiloride (DMA) treatment in the presence of benzyl alcohol might be collapsing the cellular pH gradient in the PS/NHE3 and PS/NHE1 transfectants. Intracellular pH (pH_i) was measured by the [^{14}C]-benzoic acid equilibration method (L'Allemain et al., 1984), as described in Materials and Methods. To block NHE-directed recovery of pH_i, 500 μM DMA was included in the assay buffer. The pH_i was measured in both cell lines before and after the cells were acidified by incubation in NH_4Cl saline. Acidification in the presence of DMA did not significantly affect the pH_i after 4 min, nor did the presence of 10-mM benzyl alcohol. After 4 min, in the absence of the inhibiting effects of either DMA or benzyl alcohol, partial pH_i recovery was seen. (Complete pH_i recovery in these cells is not seen for 10 to 30 min after the addition of the NH_4Cl saline. Our measurements were always performed during the linear phase of the uptake, and the assay buffer also contained 500 μM DMA so that once flux was measured no further changes in pH_i occurred.) As shown in Table 2, incubation in 10-mM benzyl alcohol did not collapse the pH gradient.

Table 3. Effect of benzyl alcohol on activity of Na, K, ATPase

Cell Line	Benzyl Alcohol	μmoles K/60 min/100 μg protein
PS120	–	8.6 ± 0.4
	+	10.0 ± 0.8*
PS/NHE-1	–	8.4 ± 0.4
	+	10.1 ± 0.3*
PS/NHE-3	–	7.9 ± 0.5
	+	9.7 ± 0.7*

Cells were incubated in fresh complete media, plus or minus 10-mM benzyl alcohol for 10 min prior to harvest. Freshly isolated membrane proteins (100 μg) were used to measure Na^+, K^+, ATPase activity by the K-stimulated phosphatase assay of Garrahan et al. (1969). The difference of phosphatase activity in the presence and absence of K^+ was defined as the K-stimulated activity. Units are μmoles K^+/60 min/100 μg protein. Values are the mean ± SEM of 3 separate experiments. *$P < 0.5$ as compared to each control by ANOVA analysis.

The Effect of Benzyl Alcohol on NHE Isoforms 1 and 3 is Specific

To further rule out a global and nonspecific modulation of membrane transport proteins by benzyl alcohol, we examined the activity of Na^+, K^+, ATPase under similar conditions. As shown in Table 3, Na pump activity in the PS fibroblasts is not inhibited by benzyl alcohol. In contrast, it showed a small, but statistically significant, increase when exposed to 10-mM benzyl alcohol. Thus benzyl alcohol appears to have a selective effect on NHEs and does not cause a generalized inhibition of all membrane transporters.

Reversibility of Inhibition

To assess whether benzyl alcohol caused irreversible damage to cellular proteins, rather than acting as a transient, potentially physiologically relevant regulatory mechanism, NHE activities were also measured after washing out the benzyl alcohol and restoring lipid fluidity to normal. This was performed in the presence and absence of cycloheximide, an inhibitor of *de novo* protein synthesis. We first determined that the fluidizing effects of 10-mM benzyl alcohol could be washed out of the cellular membranes (as shown in Table 4) with 4 × 10 min washes of complete media containing 10% serum and 2 mM glutamine. To determine the reversibility of the benzyl alcohol effect on NHE activities, one set of cells was incubated with 10-mM benzyl alcohol in media (without serum or glutamine) for 60 min; then washed 4 times (as described above) to remove all fluidizer. The control set underwent identical treatment, without fluidizer. Then both sets were incubated in NH_4Cl saline for

Table 4. The reversibility of benzyl alcohol-induced fluidity changes

	Control	Benzyl OH Treated
# of washes	r′ at 25°C	r′ at 25°C
0	0.2725 ± 0.0007	0.2680 ± 0
2	0.2740 ± 0.0014	0.2685 ± 0.0035
4	0.2740 ± 0.0001	0.2725 ± 0.0049
6	0.2745 ± 0.0007	0.2745 ± 0.0035

Intact cells were treated with 10-mM benzyl alcohol Dulbecco's modified Eagle's medium for 60 min and the *r* value of DPH at 25°C determined. This was compared with the fluidity of untreated cells (control). Both sets of cells were washed for 10 min (number of washes indicated) in complete media (DMEM + 10% serum + 2 mM glutamine) for 10 min at 25°C, and *r* values determined. ($n = 6$, mean ± SEM).

60 min (to decrease intracellular pH and activate the exchanger). Benzyl alcohol (10 mM) was added with the flux solution to half of the cells in each group, and Na uptake was measured. In parallel experiments, cycloheximide was added to the incubation, wash, and NH_4Cl saline for both groups and fluxes were measured as above. Figure 2 shows the results of these experiments (panel *A*, NHE1; panel *B*, NHE3). The first two columns in each figure reaffirm that *de novo* introduction of fluidizer significantly reduces NHE activities. (DMA-inhibitable activities in the absence of both fluidizer and protein synthesis inhibitor are set to 100%). In the presence of cycloheximide alone, NHE1 activity is slightly lower than control (but not statistically significant); while NHE-3 activity appears to be completely unaffected. However, both isoforms, when cycloheximide-treated, exhibit the same magnitude of inhibition with the addition of benzyl alcohol as do control cells. The last four columns in each graph represent the results of one hour pretreatment with benzyl alcohol, subsequent washout to restore normal fluidity, and, finally, flux measurements with or without benzyl alcohol/with or without cycloheximide. In the washout cells, in the absence of benzyl alcohol and cycloheximide, NHE1 and NHE3 activities are not significantly different from controls that had never seen the fluidizer. The addition of benzyl alcohol to the pretreated and washed cells elicits the same magnitude of inhibition as in the controls. The last two columns show the results of incubation throughout the washout experiment with cycloheximide, without and with the addition of benzyl alcohol to the final flux period. There is no statistically significant difference in the fluxes measured without benzyl alcohol among control cells, cycloheximide-treated cells, or the cells which received benzyl alcohol pretreatment and washes (with or without incubation in cycloheximide). Fresh addition of benzyl alcohol to the pretreated/washed cells (with or without cycloheximide) caused the same decrease in NHE activity seen with cells that never saw benzyl alcohol. Therefore, extended pretreatment with benzyl al-

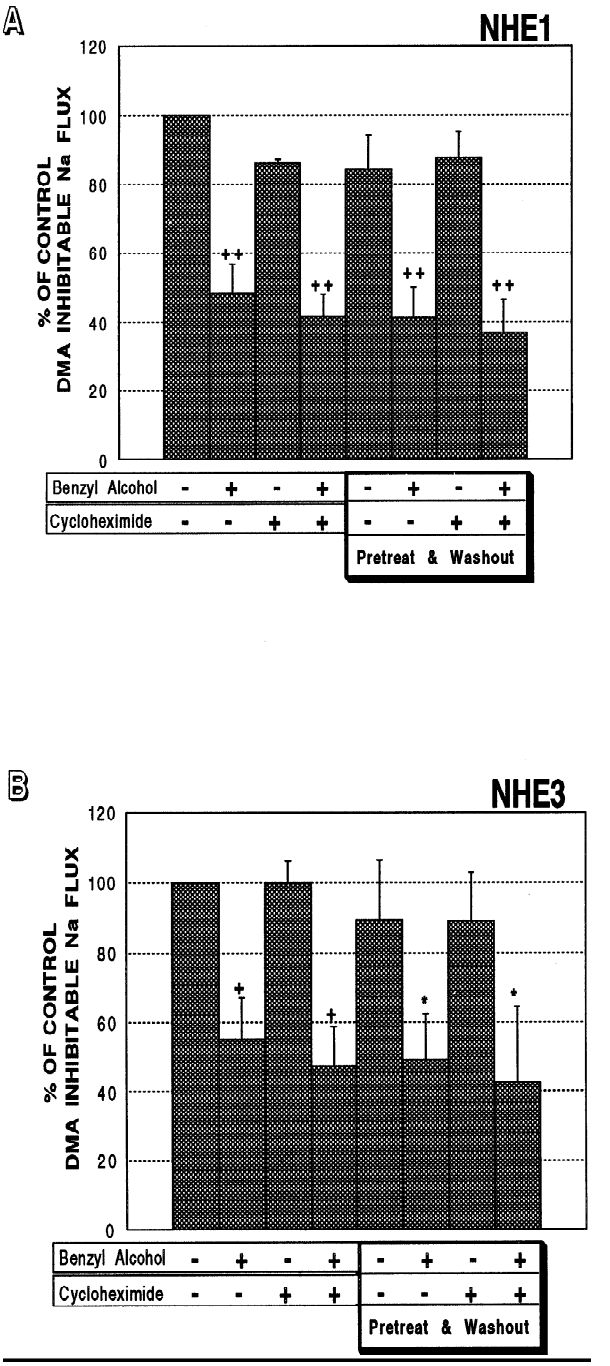

Fig. 2. The effects of benzyl alcohol on NHE activity can be reversed. (*A*) PS/NHE1 cells. (*B*) PS/NHE3 cells. Benzyl alcohol was added to a final concentration of 10 mM (denoted below each bar). Where indicated, cycloheximide was added to a final concentration of 100 μg/ml and was present in all buffers during the first 60-min acidification and 4-min flux period, during the four 10-min recovery washes, and during the second acidification and flux period. Data represent the average of four separate experiments; each point obtained in triplicate. The DMA-inhibitable Na flux of the cells *not* exposed to either the fluidizer or synthesis inhibitor (control) is set arbitrarily to 100%. All other values are represented as a percentage of that control. Statistical significance of benzyl alcohol addition to each experimental condition was calculated by ANOVA. *, $P < .05$; $^{+}$, $P < .01$; $^{++}$, $P < .001$.

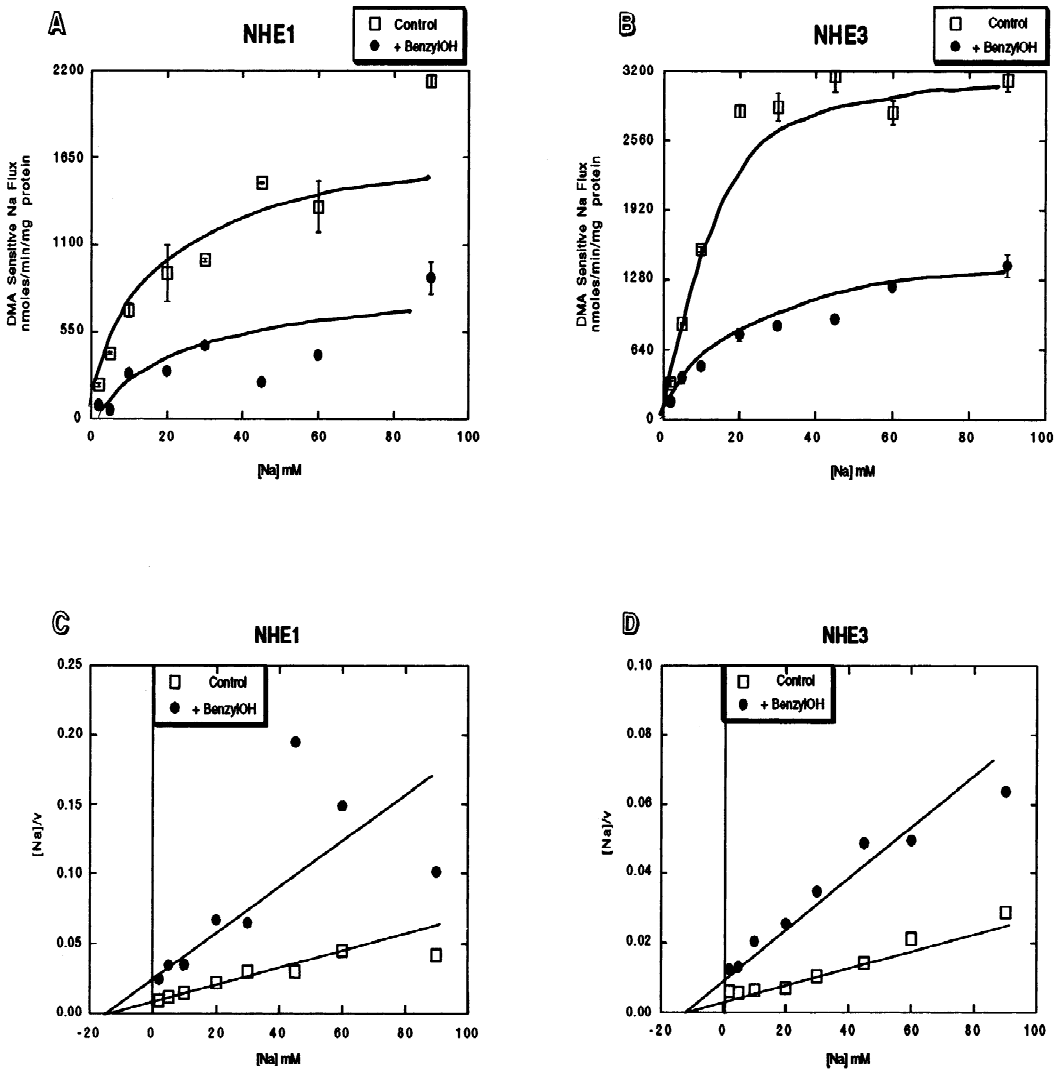

Fig. 3. The effects of the membrane fluidizer benzyl alcohol on NHE1 and 3 activities. The PS120 transfected fibroblasts were analyzed for Na uptake in the absence (□) and presence (●) of 10-mM benzyl alcohol during 4-min flux periods. Panels *A* and *B* demonstrate the results of increasing concentrations of $[Na]_o$ on dimethyl amiloride (DMA) inhibited flux activity in PS/NHE1 (*A*) and PS/NHE3 (*B*) cells. Each point represents an n of 4, 3–4 determinations per point per experiment; mean ± SD. The cells in each experiment were divided into two groups. One set was analyzed with the addition of 10-mM fluidizer in the flux solution. Specific activities were calculated as nM of Na taken up per minute per mg of protein. Panels *C* and *D* are Hanes-Woolf-Augustinsson plots of $[Na]_o$ *vs.* $[Na]_{o/v}$, to calculate the $K_{[Na]}$ and V_{Max} uptake of each NHE in the absence (□) and presence (●) of 10-mM benzyl alcohol. Panel *C* represents the results for NHE1; Panel *D* for the PS/NHE3 transfectants.

cohol did not adversely affect the activity of either NHE isoform, nor their ability to respond subsequently to the fluidizer. Thus, the inhibition of both NHE1 and NHE3 caused by benzyl alcohol is reversible and *de novo* protein synthesis is not required to restore NHE activity after washout.

Kinetics of NHE1 and NHE3 in the Presence of 10-mM Benzyl Alcohol

We examined how benzyl alcohol altered the kinetics of each exchanger by measuring the effect of increasing extracellular sodium concentrations on initial rates of uptake in the presence of fluidizer. These values were compared with fluxes of control cells from the same passage, with all fluxes measured in the same experiment. Fluxes are presented as the DMA-inhibitable component of total flux, to represent the Na uptake solely attributed to the resident NHE. Activities of both NHE1 (Fig. 3*A*) and NHE3 (Fig. 3*B*) were inhibited in the presence of 10-mM benzyl alcohol. However, V_{Max} was affected; while the K_{Na} for each (Fig. 3*C*, NHE1 and Fig. 3*D*, NHE3) was unchanged. NHE1 K_{Na} for control cells was 17.6 ± 3.8; for benzyl alcohol treated, 18.7 ± 3.3 (not statistically different). In contrast, the calculated NHE1 V_{Max} was 144.8 ± 16.1 (nmoles/min/mg) in control cells;

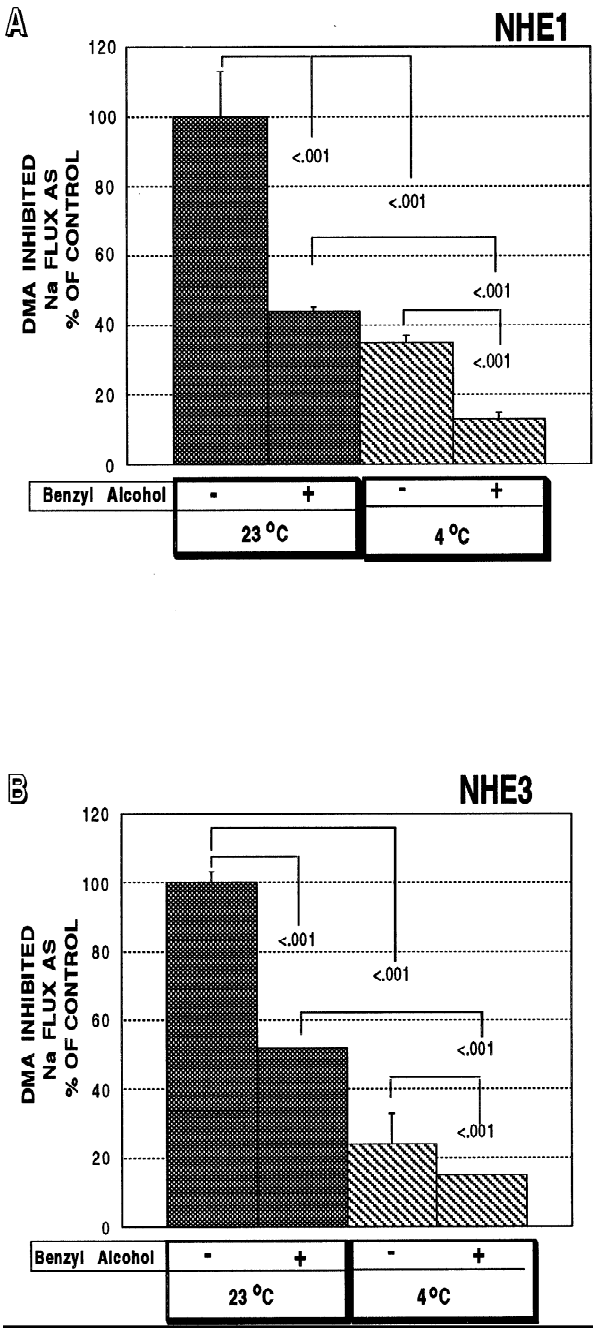

Fig. 4. Inhibition of NHE activities at 4°C in the absence and presence of benzyl alcohol. Transfectants PS/NHE1 (*A*) and PS/NHE3 (*B*) were acid loaded prior to the introduction of [^{22}Na]. Fluxes were then measured as described in Materials and Methods, at 23°C and 4°C. The Y axes reflect the DMA inhibitable component of Na uptake for each NHE as nM of Na per minute per mg protein and the values are represented as *percentage* of the flux measured at 23°C, without benzyl alcohol (arbitrarily set at 100%). Benzyl alcohol was added in parallel experiments to a final concentration of 10 mM and fluxes in those cells were also measured at 23°C and 4°C. $n = 4$. The *X*-axes indicate the variable conditions of each experiment. *P* values, shown, determined by ANOVA and brackets indicate the values which were compared to determine statistical significance.

56.2 ± 11.4 when exposed to benzyl alcohol. For NHE3, control K_{Na} (Fig. 3*D*) was 13.0 ± 0.9; with fluidizer, 11.5 ± 0.9. V_{Max} (Fig. 3*C*) in control cells was 366.1 ± 54.9 *vs.* 97.0 ± 6.6 in fluidizer treated.

Inhibition of Endo- or Exocytosis by Cold Does Not Abrogate Inhibition by Benzyl Alcohol

Because only V_{max} was affected by an increase in fluidity, we questioned if this mechanism was altering the recruitment or retention of the transporters at the plasma membrane. The NHEs were activated by preacidification in NH_4Cl saline for 60 min at 37°C. For half of the cells, an additional 15-min incubation was at 4°C. Fluxes were measured as above at room temperature (23°C) or at 4°C (buffers prechilled). The results are shown in Fig. 4*A* for NHE1 and in Fig. 4*B* for NHE3. Benzyl alcohol alone (at 23°C) inhibited NHE1 by 56%; NHE3 by 48%. Cold alone inhibited NHE1 by 65%; NHE3 by 76%. The inclusion of benzyl alcohol in the 4°C flux buffer further inhibited NHE1 to ≈12% of control; NHE3 to ≈18%.

Kinetics of the Inhibition by Amiloride and Analogues in the Presence of Benzyl Alcohol

Next we wanted to determine if increased membrane fluidity altered the kinetics of inhibition by amiloride or its analogues. As shown in Fig. 5, NHE1 is inhibited by benzyl alcohol to approximately half of the normal total flux, in the absence of any amiloride analogue. That activity is further inhibited by the addition of amiloride, DMA, EIPA or HMA. The same concentration of amiloride (0.3 mM) which inhibits the control NHE1 activity by ≈50% (Fig. 5*A*), also inhibits benzyl alcohol depressed activity by 50%. At 0.03 mM DMA (Fig. 5*B*), NHE1 activity is inhibited by 56% and benzyl-alcohol-depressed NHE1 activity by 53%. For EIPA (Fig. 5*C*) at 0.003 mM, control activity is inhibited 45%; benzyl-alcohol-depressed NHE1 activity is reduced by 47%. Only HMA (Fig. 5*D*) appears to be more effective on the control cells. At 0.01 mM, the control cells are inhibited by 57%; the benzyl alcohol cells by only 45%. For NHE3 (Fig. 6), benzyl alcohol also reduced total activity by ≈50%. Under both conditions, 0.3-mM amiloride achieved half inhibition of values without amiloride (49 and 47% respectively). DMA (Fig. 6*B*) was slightly more effective in blocking the fluidizer treated cells. At 0.03 mM, it inhibited the control cells by 21%; the benzyl alcohol cells by 31%. EIPA (Fig. 6*C*) at 0.01 mM caused 43% inhibition in control cells and 35% in the presence of fluidizer. HMA (Fig. 6*D*) was also slightly more effective on the control cells. At 0.01 mM, the control cells expressed 48% of total activity; the benzyl alcohol cells were at 62% of initial activity. Despite small variations, inhibition kinetics by amiloride and its analogues appeared to be largely unchanged in the fluidizer-treated cells.

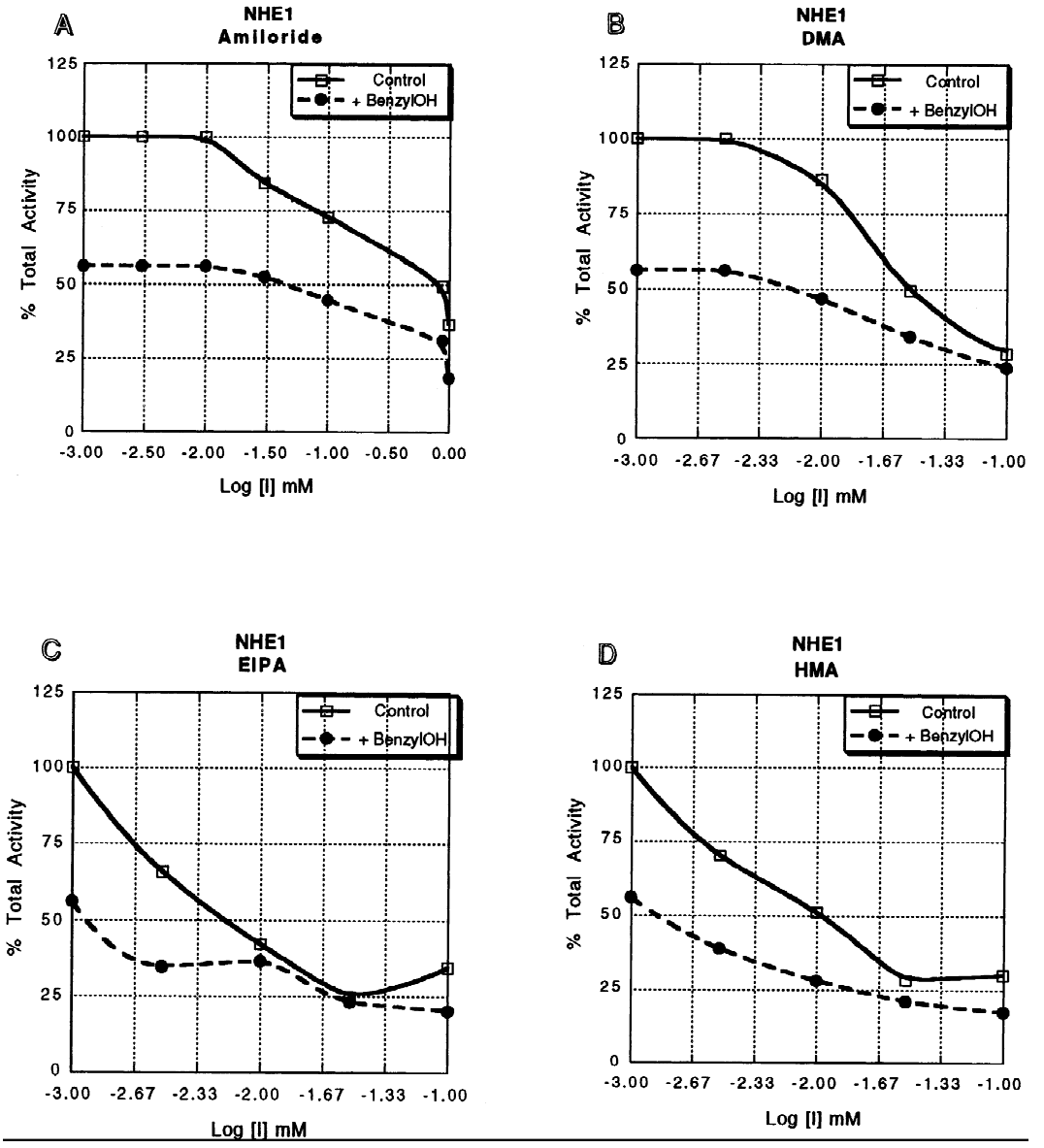

Fig. 5. Inhibition of NHE1 by amiloride and analogs in the presence of benzyl alcohol. Total activity of NHE1 in the absence of any inhibitor is arbitrarily set at 100% in each panel. The open squares (□) show relative activities of cells in increasing concentrations of amiloride or analogue. The solid circles (●) represent the activities with the addition of 10-mM benzyl alcohol. Each point is the mean ± SEM of duplicate samples in two experiments.

Discussion

This report represents the first definitive study of how alterations in plasma membrane fluidity can affect specific NHE isoforms. In this study, we have used transfected NHE-deficient fibroblasts to analyze each isoform separately. Similar studies have been done on rat intestinal brush border membrane vesicles (Brasitus et al., 1986; Dudeja, Foster & Brasitus, 1987; Dudeja et al., 1987). However, at least two NHE isoforms are endogenous to these brush borders, and those activities cannot be separated in vivo. NHE1 is localized in the basolateral membrane of polarized epithelium (Noel & Pouyssegur, 1995); NHE2 and NHE3 in the apical membrane (Biemesderfer et al., 1993; Bookstein et al., 1993; Kuwahara et al., 1994; Orlowski et al., 1992). The present study also differs in the use of intact cells, whereas brush border membrane vesicles have an incomplete cytoskeleton and disrupted transcellular gradients. Although we observed no change in affinity to sodium (K_{Na}) for either NHE1 or NHE3, benzyl alcohol significantly reduced the V_{max} of both in a dose-dependent fashion. With measurements of pH_i before and after benzyl alcohol treatment, we determined that a collapse of the pH gradient

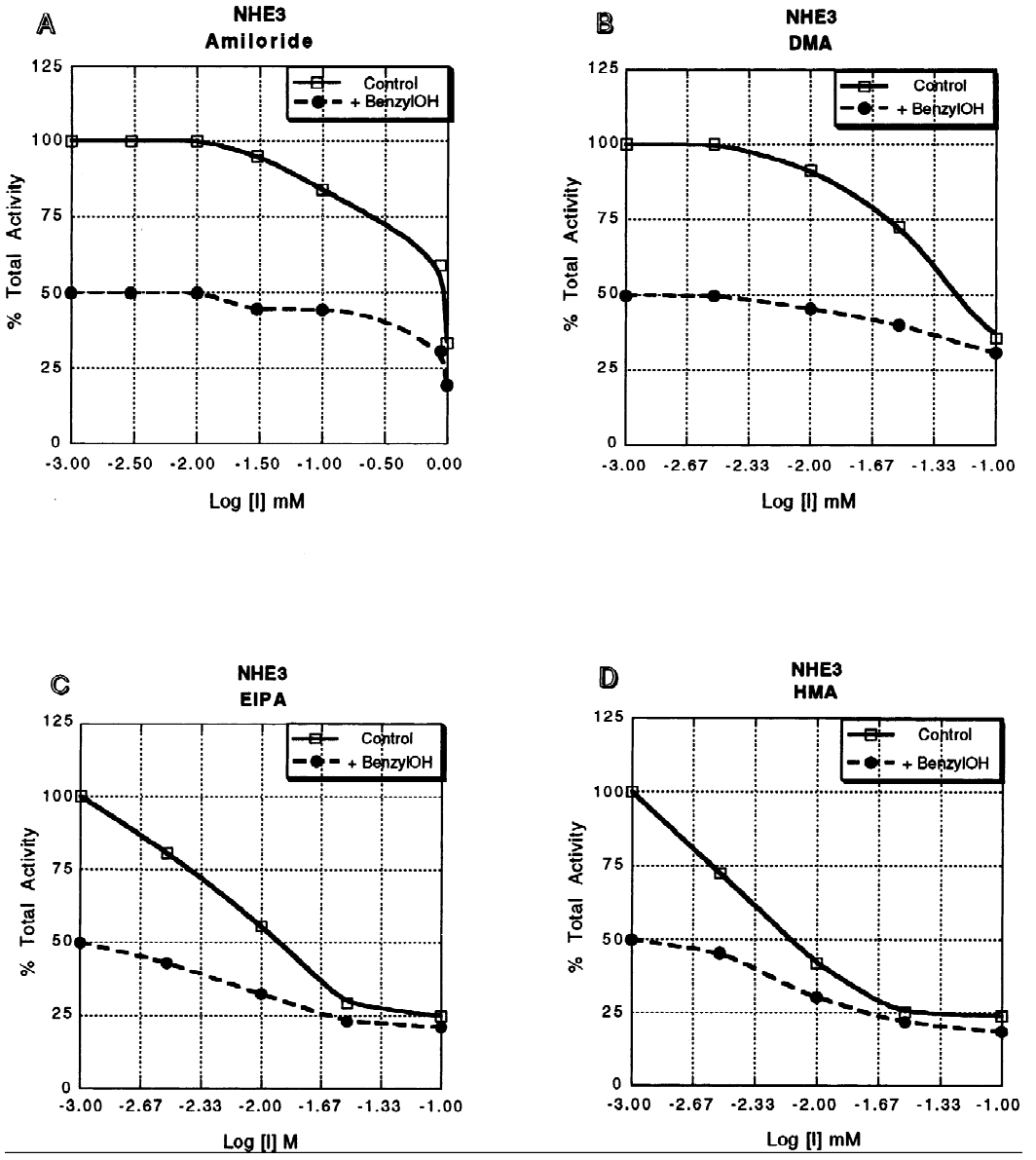

Fig. 6. Inhibition of NHE3 by amiloride and analogues in the presence of benzyl alcohol. Total activity of NHE3 in the absence of any inhibitor is arbitrarily set at 100% in each panel. The open squares (□) show relative activities of cells in increasing concentrations of amiloride or analogue. The solid circles (●) represent the activities with the addition of 10-mM benzyl alcohol. Each point is the mean ± SEM of duplicate samples in two experiments.

was not modulating NHE activities. Also, this effect was not caused by irreversible damage to the proteins, as confirmed by the reversibility of repression demonstrated in the washout experiments. Further, we established that restoration of the NHEs to full activity after treatment with fluidizer and its subsequent removal did not require *de novo* protein synthesis.

Significantly, the inhibitor effect also appeared to be selective, as another membrane ion transporter protein, Na^+, K^+, ATPase, was not similarly affected. In fact, Na pump activity was stimulated slightly by benzyl alcohol, suggesting that fluidity alterations alone are not sufficient to have a global effect on all integral membrane proteins. Under the conditions which significantly inhibited NHE activity in this study, Na pump activity showed a slight increase. Yet Na pump activity can be modulated by increased membrane fluidity under specific conditions in other cell types. Molitoris et al. (1992) reported that increasing membrane fluidity of brush-border membrane vesicles isolated from renal proximal tubule *after* ischemia further augmented the already elevated Na pump activity. Treatment of control vesicles with the same fluidizer had no effect on activity. This would suggest that a combination of lipid composition and cell

specific regulators may determine the response of membrane proteins to fluidity changes.

Benzyl alcohol-induced increases in membrane fluidity did not appear to significantly alter the conformation of the amiloride binding site in either NHE1 or NHE3. In the presence of the fluidizer, both NHE1 and NHE3 displayed dose responses similar to the control for amiloride and its analogues. Half maximal inhibition (based on starting activity in the absence of analogue) was reached at approximately the same concentration in control and benzyl alcohol treated. This also suggests, as does the kinetic data, that the fluidizer does not impede the uptake of Na^+ in the same way that amiloride or its analogues do.

To understand the mechanism by which benzyl alcohol modulates NHE activity, we tested the hypothesis that the fluidizer inhibits translocation of NHE-bearing transport vesicles to the plasma membrane, or stimulates endocytosis of those already there. At 4°C, vesicle movement, ion transport and enzyme activity are slowed. As expected, activities of both NHE isoforms in the cold were significantly inhibited. However, the additive inhibitory effect of benzyl alcohol at 4°C argues against the hypothesis that this fluidizer lowers V_{max} solely by affecting the vesicle transport of exchangers. The further depression of activity in the 4°C-preincubated cells (containing benzyl alcohol only in the flux buffer) suggests modulation of NHE activity may occur by two different mechanisms. Our data do not allow us to definitively rule out vesicle movement as a mechanism by which benzyl alcohol regulates NHE activity. However, it is also possible that this fluidizer lowers V_{max}, not solely by any change in vesicle movement, but by directly or indirectly altering the active state of the NHE protein.

In summary, this report provides evidence that increased membrane fluidity inhibits two Na/H exchangers, NHE1 and NHE3, and presents detailed kinetic characterization of the effects of increased membrane fluidity on both isoforms. NHE1 is the constitutively expressed regulator of pH_i and cell volume; NHE3 plays a significant role in transcellular transport of Na in the gut. Future studies will examine the mechanism by which lipid environment regulates the activities of these isoforms.

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