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Effect of Trace Levels of Nigericin on Intracellular pH and Acid-Base Transport in Rat Renal Mesangial Cells

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Abstract. Nigericin is an ionophore commonly used at the end of experiments to calibrate intracellularly trapped pH-sensitive dyes. In the present study, we explore the possibility that residual nigericin from dye calibration in one experiment might interfere with intracellular pH (pH_i) changes in the next. Using the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), we measured pH_i in cultured rat renal mesangial cells. Nigericin contamination caused: (i) an increase in acid loading during the pH_i decrease elicited by removing extracellular Na^+, (ii) an increase in acid extrusion during the pH_i increase caused by elevating extracellular $[K^+]$, and (iii) an acid shift in the pH_i dependence of the background intracellular acid loading unmasked by inhibiting Na-H exchange with ethylisopropylamiloride (EIPA). However, contamination had no effect on the pH_i dependence of Na-H exchange, computed by adding the pH_i dependencies of total acid extrusion and background acid loading. Nigericin contamination can be conveniently minimized by using a separate line to deliver nigericin to the cells, and by briefly washing the tubing with ethanol and water after each experiment.

Key words: Acid extrusion — Acid loading — BCECF — Dye — Ionophore — pH_i

Introduction

Nigericin, a polyether antibiotic from the fungus *Streptomyces hygroscopicus*, is a carboxylic ionophore that exchanges H^+ for a cation such as K^+ or Na^+ across cell membranes [for review, *see* ref. 16]. The ionophore has a lower affinity for Na^+ than for K^+ [15]. Thomas et al. [20] were the first to use nigericin, added to the extracellular solution, to set intracellular pH (pH_i) equal to extracellular pH (pH_o). This approach has become widely used for calibrating intracellularly trapped pH-sensitive dyes, as well as for other applications in which it is critical to control pH_i. When cells are exposed to a Na^+-free solution containing K^+ and nigericin, the ionophore predominantly exchanges H^+ for K^+, so that the H^+ and K^+ gradients across the membrane equalize. If one chooses a value for $[K^+]_o$ that is the same as $[K^+]_i$, then nigericin will clamp $[H^+]_i$ to $[H^+]_o$; that is, pH_i will equal pH_o. Some of the potential problems of using nigericin for the purpose of calibrating pH-sensitive indicators have been discussed by Chaillet et al. [8], Boyarsky et al. [3–5], and by Bevensee and Boron [1].

Richmond and Vaughan-Jones [17] have recently suggested another potential problem with using nigericin to calibrate pH-sensitive dyes. Trace amounts of nigericin remaining in the experimental apparatus after a calibration procedure can contaminate cells in the following experiment, complicating the interpretation of pH_i data. The residual ionophore can masquerade as an endogenous monovalent cation exchanger, facilitating either Na-H exchange or K-H exchange, depending on the prevailing ionic concentrations. Indeed, Richmond and Vaughan-Jones [17] have demonstrated that nigericin contamination can account for data previously used to support the existence of a K-H exchanger. Moreover, they showed that trace levels of nigericin in an inadequately washed perfusion system enhance acid loading in carotid-body type I cells. The Vaughan-Jones' group avoids nigericin contamination by washing the perfusion system with a strong detergent for an impractically long period of time, and by calibrating experiments only once a month [13, 19].

In the present study on mesangial cells cultured from rat kidney, we also found that residual nigericin from the dye calibration in one experiment can interfere with pH_i

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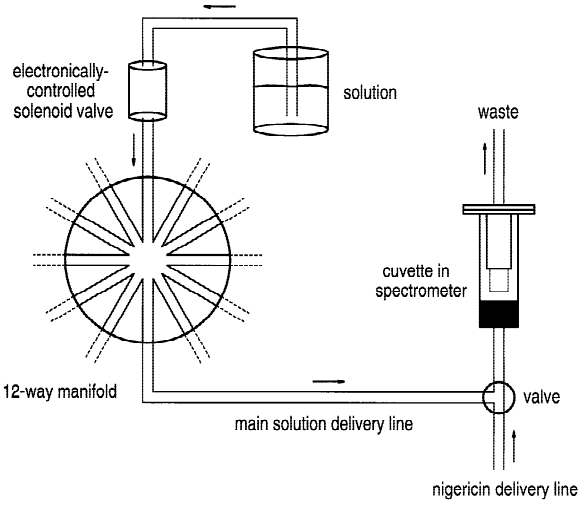


Fig. 1. During experiments, solutions were delivered to cells in a cuvette through a main solution delivery line attached to a 12-way manifold made of Plexiglass®. The tubing was made of Saran® or Tygon®, and was attached to the manifold and to the chamber via short lengths of stainless steel tubing press fit into the plastic. In some experiments, the nigericin-containing solution was delivered through the manifold, whereas in others, it was introduced via a separate nigericin delivery line spliced to the main solution delivery line.

changes in the next experiment. However, we found that nigericin contamination can easily be minimized by delivering the nigericin through dedicated tubing, and washing this tubing with ethanol and water after each experiment. Moreover, even if one takes no special precautions to reduce the contamination, residual nigericin does not alter the pH_i dependence of the appropriately computed Na-H exchange rate.

Materials and Methods

Solutions

The standard HEPES-buffered solution contained (in mM): 125 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4, 32 HEPES, 10.5 glucose, and titrated to 7.4 at 37°C with NaOH. All experiments were performed in the nominal absence of CO_2/HCO_3^-. In the Na^+-free solutions, the Na^+ substitute was *N*-methyl-D-glucammonium ($NMDG^+$). In the 40 mM KCl solution, 35 mM NaCl was replaced by an equal concentration of KCl. In the 20 mM NH_3/NH_4^+ solution, 20 mM NaCl was replaced by an equal concentration of NH_4Cl. In the calibration solution containing 10 μM nigericin, NaCl was replaced by 105 mM KCl and $NMDG^+$. The acetoxymethyl ester of BCECF (BCECF-AM) was obtained from Molecular Probes (Eugene, OR). Nigericin and albumin were obtained from Sigma Chemical (St. Louis, MO). Ethanol was obtained from Pharmco Products (Brookfield, CT). Decon90® was obtained from Decon Laboratories Limited (E. Sussex, U.K.). EIPA was obtained from either E.J. Cragoe, Jr. (Nacogdoches, TX) or Research Biochemicals (Natick, MA).

Cell Preparation

The mesangial cells were prepared as previously described [6,10,14]. Briefly, cells from passage 3–7 were plated onto glass coverslips and grown at 37°C, 5% CO_2/balance air in Dulbecco's modified Eagle's medium (D-MEM; GIBCO BRL, Life Technologies, Gaithersburg, MD) supplemented with 100 units/ml penicillin/streptomycin (GIBCO BRL, Life Technologies), 5 μg/ml of insulin/transferrin/selenious acid (ITS; Becton Dickinson Labware, Bedford, MA), and 10% fetal calf serum (Gemini Bioproducts, Calabasas, CA or GIBCO BRL, Life Technologies). Cells were rendered quiescent by incubating them in a medium containing only 0.5% FCS, beginning 16–18 hr prior to experiments.

Measurement of pH_i

Our technique for measuring pH_i has been previously described [12]. Briefly, the flow-through cuvette containing a coverslip was placed in a SPEX Fluorolog-2 spectrofluorometer (model CM1T10E; SPEX Industries, Edison, NJ). Cells loaded with BCECF were alternately excited with pH-sensitive 502 nm light (I_{502}) and relatively pH-insensitive 440 nm light (I_{440}). The emission wavelength was 526 nm. The fluorescence-excitation ratio (I_{502}/I_{440}) was converted to a pH_i value using the high-K^+/nigericin technique [20], as modified for a single-point calibration [6].

Changing Solutions and Washing the Perfusion System

The perfusion system is shown in Fig. 1. As many as 11 solutions were delivered via Tygon® tubing (Fisher Scientific, Pittsburgh, PA) into 11 ports of a 12-way Plexiglass® manifold. The twelfth port was connected to the main solution delivery line, which carried fluid to the cuvette. Part of this main solution delivery line was either Saran® or Tygon® tubing, and part was stainless steel tubing surrounded by a water jacket, so that the solution entering the cuvette was ~37°C. The main solution delivery line terminated at the base of the cuvette, which was housed in the spectrometer. In some experiments, a separate nigericin delivery line of Tygon® tubing was connected to the main solution delivery line via a 3-way plastic valve. During a single experiment, electronically controlled solenoid valves (General Valve, Fairfield, NJ) controlled the flow of solution into as many as 11 different inlet ports of the manifold. Solutions were delivered at ~5 ml/min to an ~1-ml cuvette. All experiments were performed at 37 ± 1°C. A full description of each of the wash protocols of the perfusion system is provided in the Table. The line washes described in the Table also cleansed the associated solenoid valves and inlet ports of the manifold. Washes were performed at room temperature.

The main solution delivery line contained Saran® tubing in early experiments and Tygon® tubing in later ones. Based on our Na^+-removal assay (*see* below), the amount of nigericin contamination that could be removed by a "Full EtOH wash" was no different with Saran® *vs.* Tygon® tubing.

Statistics

Data are reported as mean ± SEM. Levels of significance were assessed using the unpaired Student's *t*-test or one-way analysis of variance. A P value < 0.05 was considered significant.

Results

Washing Nigericin from the Perfusion System with Ethanol or Albumin

The Acid-Loading Rate Elicited by Removing External Na^+ or the Acid-Extrusion Rate Caused by Raising External K^+ is an Index of Nigericin Contamination

Because nigericin exchanges H^+ for K^+ (and less so, H^+ for Na^+) across cell membranes, we speculated that nigericin contamination would contribute to both the pH_i decrease elicited by removing external Na^+, and the pH_i increase caused by increasing external K^+.

Figure 2*A* illustrates the results from two experiments performed on the same day. Before each experiment, we cleansed the perfusion system using either the "Full EtOH wash" protocol, or the "Minimal wash" protocol (*see* Table). In both experiments, replacing external Na^+ with $NMDG^+$ elicited a decrease in pH_i (*ab* and *ab′*, respectively), probably reflecting reversal of Na-H exchange and unmasking of background acid loading. Indeed, the endogenous Na-H exchanger in these mesangial cells is active at a pH_i of ~7.1 (*see below*). In the continued absence of external Na^+, raising external K^+ from 5 to 40 mM caused an increase in pH_i that was partially reversible in both experiments (*bcd* and *b′c′d′*). A similar segment-*bc* (or segment-*b′c′*) pH_i increase elicited by raising extracellular K^+, termed a depolarization-induced alkalinization (DIA), was first observed using microelectrodes (i.e., no nigericin contamination) in salamander proximal-tubule cells [18], and later in other preparations, particularly invertebrate and mammalian glial cells [*see* ref. 9].

There are two noteworthy points regarding Fig. 2*A*. First, the pH_i decreased at a greater rate and to a greater extent when we replaced Na^+ with $NMDG^+$ in the "Minimal wash" experiment than in the "Full EtOH wash" experiment. Comparing the mean initial rates of acidification, we found that the "Full EtOH wash" reduced the acidification rate by 43%. This faster and larger pH_i decrease in the "Minimal wash" experiment is consistent with the presence of residual nigericin, which would facilitate greater exchange of internal Na^+ for external H^+ (*see* Nigericin/Model 1, Fig. 2*A*). It is also consistent with an unmasking of nigericin-mediated K-H exchange.

The second noteworthy point in Fig. 2*A* is that pH_i increased more rapidly and to a greater extent when we increased $[K^+]_o$ to 40 mM in the "Minimal wash" experiment than in the "Full EtOH wash" experiment. Comparing the mean initial rates of alkalinization, we found that the "Full EtOH wash" reduced the alkalinization rate by 35% (Fig. 2*B*). This faster and larger pH_i increase in the "Minimal wash" experiment again is consistent with the presence of residual nigericin, which would facilitate greater exchange of external K^+ for internal H^+ (*see* Nigericin/Model 2, Fig. 2*A*). However, part of the difference may have been due to differences in the prevailing pH_i. On average, the pH_i at point *b*/*b′* was ~0.1 higher ($P = 0.009$) in the "Full EtOH wash" experiments than in the "Minimal wash" experiments. Nevertheless, even if we ignore a potential pH_i effect, the dpH_i/dt differences in Fig. 2*A* are smaller for increasing $[K^+]_o$ than for re-

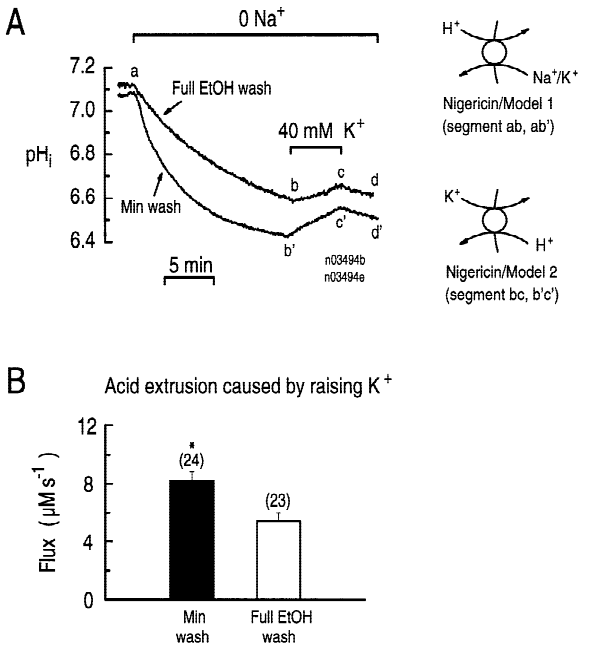


Fig. 2. The rate of acidification caused by removing Na^+, and the rate of alkalinization elicited by raising K^+ are greater in "Minimal wash" experiments than in "Full EtOH wash" experiments. (*A*) In both experiments, the cells had an average pH_i of ~7.1 prior to point *a*. Removing external Na^+ caused pH_i to decrease at a faster rate and to a greater extent in the "Minimal wash" experiment (*ab′*) than in the "Full EtOH wash" experiment (*ab*). In the continued absence of external Na^+, elevating external K^+ from 5 to 40 mM elicited an increase in pH_i that was faster and somewhat larger in the "Minimal wash" experiment (*b′c′*) than in the "Full EtOH wash" experiment (*bc*). In both experiments, returning the cells to the 5 mM K^+, Na^+-free solution caused the pH_i to decrease (*cd* and *c′d′*). (*B*) A summary of the mean acid-extrusion rates elicited by raising external K^+ in experiments similar to those shown in *A*. The "Full EtOH wash" experiments are compared to "Minimal wash" experiments performed within the same week. The data are from a subset of the experiments in which we also calculated the average acid-loading rate elicited by removing external Na^+ (first pair of bars in Fig. 3*A*). Acid-loading or acid-extrusion rate is the product of dpH_i/dt and the ability of the cells to buffer changes in H^+ (i.e., the intrinsic intracellular buffering power, β_I, which is determined in the nominal absence of CO_2/HCO_3^-). The pH_i dependence of β_I for individual mesangial cells, determined previously [6], is described by the equation: $\beta_I = -7.8 \times pH_i + 61.9$. *n* values are given in the parentheses above each point. *$P < 0.0001$ (unpaired Student's *t*-test).

Table. Wash protocols of perfusion system

Wash protocol	Lines washed	Lines replaced	Wash solution and duration (min)						
			Decon®	H_2O	Alb[a]	H_2O	EtOH[b]	H_2O	Std[c]
Minimal	Std HEPES	None	—	—	—	—	—	—	5
Full EtOH	All	None	—	2	—	—	5	5	1–2
Full albumin	All	None	—	2	14[d]	5	5	5	1–2
Partial EtOH	Calibration	None	—	2	—	—	5	5	1–2
Partial albumin	Calibration	None	—	2	14[d]	5	5	5	1–2
Decon®	All	None	1440	—	—	—	—	2880	1–2
Decon® Plus	All	All	1440	—	—	—	—	2880	1–2
2-line/Part EtOH[e]	Calibration	None	—	2	—	—	5	5	1–2

[a] 2% Bovine serum albumin;
[b] 75% Ethanol;
[c] Standard HEPES-buffered solution;
[d] 2-min rinse, then 10-min soak, finally 2-min rinse again;
[e] The nigericin solution was delivered via dedicated tubing that bypassed the manifold (*see* Fig. 1).

moving Na^+. Therefore, in subsequent experiments, we focused on removing Na^+.

Other Wash Protocols are as Effective as a "Full EtOH Wash"

Figure 3*A* summarizes the results of five wash protocols for the Na^+-removal assay (*ab* in Fig. 2*A*). Each pair of bars in the figure compares the results for a "Full EtOH wash" with one of the other four protocols described in the Table. In each case, we compared only experiments done in the same week. Comparing 33 "Minimal wash" experiments and 33 week-matched "Full EtOH wash" experiments (first pair), we see that the "Full EtOH wash" reduced the net acid influx produced by removing Na^+ by 43% ($P < 0.0001$). The "Full albumin wash," in which we washed all the lines with 2% albumin in addition to the usual 75% ethanol wash, was no more effective than the simple "Full EtOH wash" (second pair). Because albumin has been used to scavenge nigericin from cell membranes [11], we expected the albumin wash to remove more of the nigericin, assuming that some nigericin continued to contaminate the system after an ethanol wash.

In the "Partial EtOH wash," we used only ethanol to wash only the line carrying the nigericin to the manifold, as well as the main solution delivery line and cuvette (third pair). In the "Partial albumin wash," we used albumin and then ethanol to wash only the line carrying the nigericin to the manifold, as well as the main solution delivery line and cuvette (fourth pair). Neither the "Partial EtOH wash" nor the "Partial albumin wash" produced fluxes significantly different from the matched "Full EtOH washes." Therefore, washing only the cuvette and the tubing used to deliver the nigericin solution to the cuvette appears to be no worse than the more comprehensive "Full EtOH wash."

Washing Nigericin from the Perfusion System with a Strong Detergent

One of the ways Richmond and Vaughan-Jones [17] assessed nigericin contamination in carotid-body glomus cells was by simultaneously (i) removing extracellular Na^+ and (ii) adding amiloride to the extracellular fluid in a sodium-free amiloride ("SFA") solution. In experiments conducted immediately after an experiment involving a calibration with nigericin, the SFA solution produced a large and rapid acidification. We presume that this acidification is mainly due to the cessation of nigericin-mediated exchange of external Na^+ for internal H^+, and its replacement by nigericin-mediated exchange of external H^+ for internal Na^+ and K^+. When these authors extensively washed their perfusion system with Decon®, a strong detergent, and then distilled water, they found that the rate of the pH_i decrease produced by the SFA solution was only one-thirtieth the previous value.

Given the experience of Richmond and Vaughan-Jones with their Decon® wash, we tested the possibility that part of the flux remaining after any of the washes in Fig. 3*A*, except for the "Minimal wash," was due to residual nigericin, reflecting an exchange of external H^+ for internal Na^+ and K^+. One approach ("Decon® wash," Table) was to wash the entire perfusion system with Decon® for one day, and then rinse with water for two days. In other experiments ("Decon® Plus wash," Table), we replaced all the Saran® or Tygon® tubing of the perfusion system, and then followed the "Decon® wash" protocol to cleanse the manifold and the cuvette.

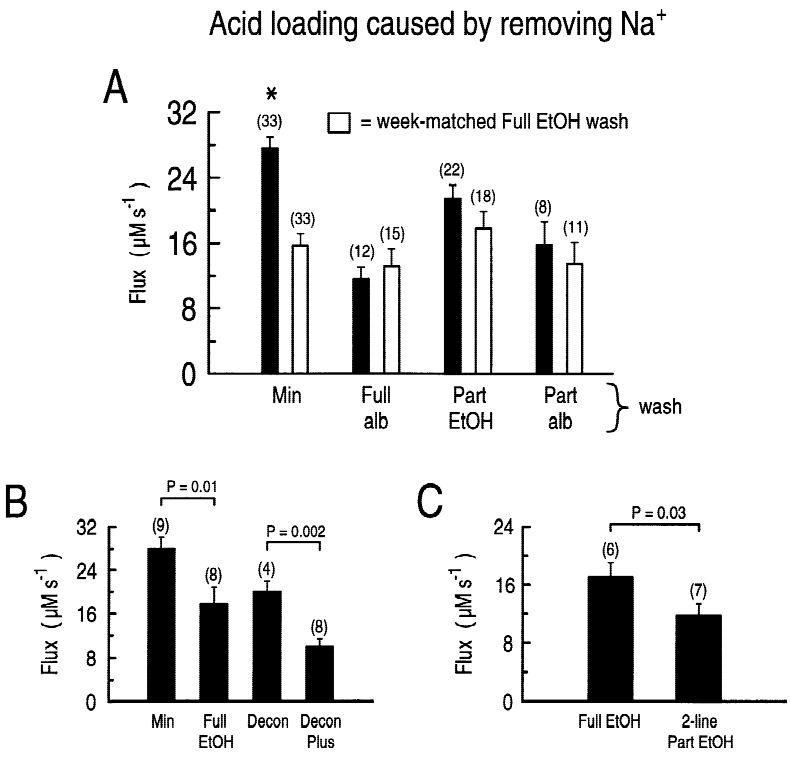


Fig. 3. The acid-extrusion rate elicited by removing external Na^+ in "Full EtOH wash" experiments can be reduced further by either replacing the perfusion lines, or using a separate line to deliver nigericin to the cells. (*A*) Wash protocols that are as effective as a "Full EtOH wash." The first pair of bars summarizes the results from experiments similar to those shown in Fig. 2*A*. The mean acid-loading rates obtained with the other wash protocols (Table) were similar to the mean rate obtained with the week-matched "Full EtOH" wash protocol ($P > 0.05$, unpaired, Student's *t*-test). *n* values are given in the parentheses above each bar. *$P < 0.001$ (unpaired Student's *t*-test), compared to the corresponding week-matched "Full EtOH wash." (*B*) Replacing the perfusion lines and extensively washing the perfusion system with the strong detergent Decon® ("Decon® Plus wash"). Bar 2 is less than bar 1 ($P = 0.01$), and bar 4 is less than bar 3 ($P = 0.002$). However, bar 2 and bar 3 are not different ($P = 0.28$). *n* values are given in the parentheses above each bar. The bars were compared using one-way analysis of variance. (*C*) Using a separate line to deliver nigericin to the cells, and washing the tubing with ethanol and water after each experiment ("2-line/Part EtOH wash"). *n* values are given in the parentheses above each bar. The two bars were compared using an unpaired Student's *t*-test.

Figure 3*B* summarizes the results of paired experiments[1] in which we compared the efficacy of a "Minimal wash," a "Full EtOH wash," a "Decon® wash" and a "Decon® Plus wash." When following either the "Decon® wash" or the "Decon® Plus wash" protocol, we avoided exposing the perfusion system to nigericin between experiments; we performed a nigericin calibration only in the final experiment of the day. We used the calibration results from this final experiment to convert I_{502}/I_{440} to pH_i values for all experiments of that day.

In experiments similar to those shown in Fig. 2*A*, the mean acid-loading rate caused by removing external Na^+ in eight "Full EtOH wash" experiments (second bar, Fig. 3*B*) was 36% less than the acid-loading rate in nine "Minimal wash" experiments (first bar); the difference was statistically significant ($P = 0.01$). These data thus confirm the results summarized by the first pair of bars in Fig. 3*A*. The "Decon® wash" protocol (third bar, Fig. 3*B*) did not further reduce the acid-loading rate. However, in eight "Decon® Plus wash" experiments (fourth bar), in which we also replaced the tubing, the mean acid-loading rate was significantly less ($P = 0.002$) than the mean rate in the four "Decon® wash" experiments in which the perfusion tubing was not replaced. The flux in the "Decon® Plus wash" experiments was ~50% of that in the simpler "Decon® wash" experiments, and ~56% of the flux in the "Full EtOH wash" experiments. Therefore, although a "Decon® wash" by itself is no better than a "Full EtOH wash" in reducing nigericin contamination, the additional maneuver of replacing the tubing reduced the acid-loading rate by about half.

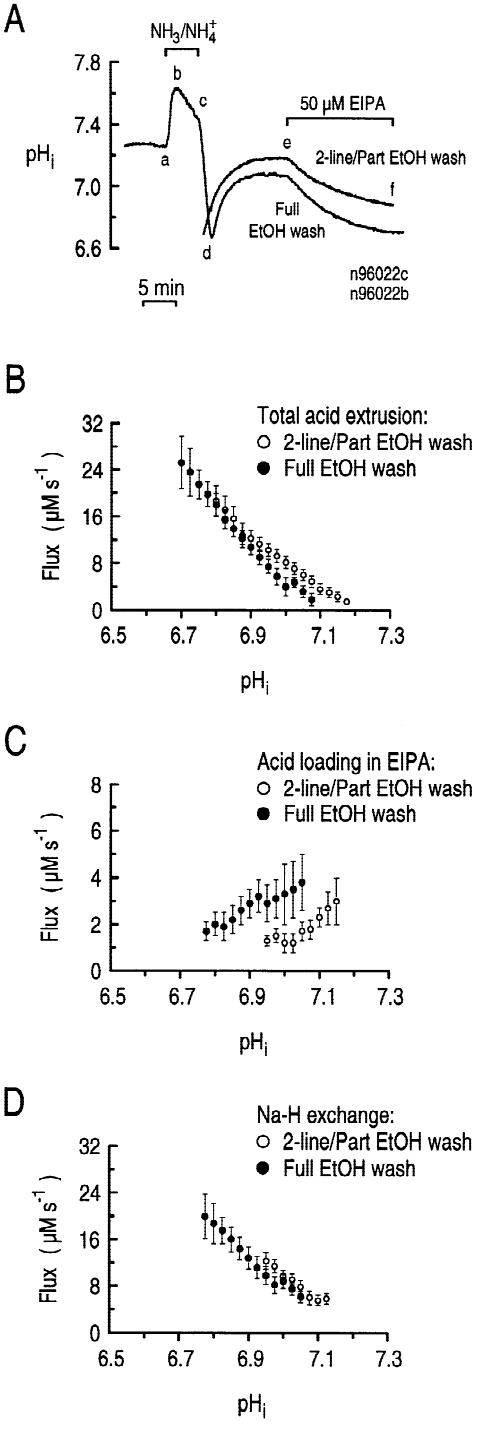
Isolating the Perfusion Line Delivering Nigericin to the Cuvette

From the results summarized in Fig. 3*B*, we conclude that a fraction of the nigericin used in a calibration procedure can remain in the perfusion system, even after extensive washing of the system with a strong detergent. Although replacing all of the perfusion tubing can minimize the nigericin contamination, this procedure is arduous and time consuming. We therefore examined an alternative approach: Disconnect the tubing that carries the nigericin solution to the manifold, and splice it into the main solution delivery line, just before it enters the stainless-steel tubing on its way to the cuvette (Fig. 1). In this arrangement, the dedicated nigericin line bypasses the manifold, thereby greatly reducing the surface area of tubing and Plexiglass® (i.e., the manifold) exposed to the ionophore.

Our approach was to perform a "Full EtOH wash"

[1] These experiments were all performed over three, two-day periods. Each two-day period included a representative sample of all four protocols.

and then to remove extracellular Na^+, using the protocol introduced in Fig. 2*A*. In the experiment preceding the one in which we actually gathered the data, the nigericin solution either flowed into the manifold ("Full EtOH wash," Fig. 3*C*), or bypassed the manifold and flowed via dedicated tubing into the main solution delivery line ("2-line/Part EtOH wash," Fig. 3*C*). The latter is the "2-line/Part EtOH wash" protocol described in the Table. As summarized in Fig. 3*C*, the mean acid-loading rate with two lines was 32% less than the mean rate with one line; the difference was statistically significant ($P = 0.03$). We did not compare the "2-line/Part EtOH wash" (bar 2, Fig. 3*C*) with the "Decon® Plus wash" (bar 4, Fig. 3*B*) in paired experiments. However, bar 1 in Fig. 3*C* ("Full EtOH wash") is the same protocol as bar 2 in Fig. 3*B* ("Full EtOH wash"). The fluxes represented by these two bars are virtually identical (17.8 *vs.* 17.1 μM sec^{-1}) and do not differ statistically. Bar 2 in Fig. 3*C* ("2-line/Part EtOH wash") represents a flux of 11.7 μM sec^{-1}, which is not statistically different from the flux of 9.9 μM sec^{-1} represented by bar 4 in Fig. 3*B* ("Decon® Plus wash"). Thus, it appears that using a simple ethanol wash in conjunction with a dedicated nigericin line is nearly as effective, if not as effective, as replacing all of the tubing and then washing with detergent (1 day) and water (2 days).



The Influence of Nigericin Contamination on Acid Loading and Acid Extrusion During the pH_i Recovery from an Acid Load

According to Fig. 3*A*, several of the washing procedures eliminate some of the nigericin contamination, whereas according to Fig. 3*B* and *C*, either the "Decon® Plus wash" or the "2-line/Part EtOH wash" protocols eliminate even more of the contamination. In Figs. 4 and 5, we assess the extent to which the different degrees of nigericin contamination can influence the acid-loading and acid-extrusion rates computed from experiments designed to determine the pH_i dependence of Na-H ex-

Fig. 4. Contaminating nigericin in "Full EtOH wash" experiments increases background acid loading of mesangial cells, but has no effect on the pH_i dependence of Na-H exchange. (*A*) The pH_i recovery from an acid load, and the pH_i decrease elicited by 50 μM EIPA in a "Full EtOH wash" and a "2-line/Part EtOH wash" experiment. For the "2-line/Part EtOH wash" experiment, we only show the pH_i record following the acid load. When the cells were exposed to 20 mM NH_3/NH_4^+, the pH_i increased rapidly (*ab*), and then declined more slowly (*bc*). Removing the external NH_3/NH_4^+ elicited a rapid decrease in pH_i (*cd*), followed by a slower recovery (*de*). After this recovery, applying 50 μM EIPA caused the pH_i to decline slowly (*ef*). (*B*) The pH_i dependence of total acid extrusion (φ_E) in "Full EtOH wash" experiments (closed circles) and "2-line/Part EtOH wash" experiments (open circles). φ_E is the product of the rate of the pH_i recovery from an acid load (e.g., segments *de* in *A*) and the pH_i dependence of β_I (*see* legend, Fig. 2*B*). For *B–D*, each point represents an *n* of three or more. (*C*) The pH_i dependence of background acid loading (φ_{EIPA}) in "Full EtOH wash" experiments (closed circles) and "2-line/Part EtOH wash" experiments (open circles). φ_{EIPA} is the product of the rate of the pH_i decrease elicited by EIPA (e.g., segments *ef* in *A*) and the pH_i dependence of β_I. Note that the data in *C* represent the same wash protocols as in *B*. (*D*) The pH_i dependence of Na-H exchange ($\varphi_{Na\text{-}H}$) in "Full EtOH wash" experiments (closed circles) and "2-line/Part EtOH wash" (open circles) experiments. The pH_i dependencies of $\varphi_{Na\text{-}H}$ for the two wash protocols are the sum of the pH_i dependencies of φ_E (in *B*) and φ_{EIPA} (in *C*).

change. In Fig. 4, we compare the "2-line/Part EtOH wash" with the "Full EtOH wash," and in Fig. 5, we compare the "2-line/Part EtOH wash" with the "Minimal wash." In each case, we examined three parameters [7]: (i) the pH_i dependence of "total acid extrusion" during the pH_i recovery from an acid load (φ_E), (ii) the pH_i dependence of "background acid loading" during the pH_i decrease elicited by applying EIPA (φ_{EIPA}), and (iii) the pH_i dependence of the Na-H exchange rate ($\varphi_{Na\text{-}H} = \varphi_E + \varphi_{EIPA}$ at the same pH_i).

The results from two experiments performed on the same day are shown in Fig. 4*A*. Before each experiment, we cleansed the perfusion system using either the "Full EtOH wash" protocol or the "2-line/Part EtOH wash" protocol. We used the NH_4^+-prepulse technique [2] to acid load the cells (*abcd*). In both experiments, the pH_i recovered from the acid load (*de*). Subsequently, applying 50 μM EIPA, a potent inhibitor of Na-H exchange, elicited a gradual decrease in pH_i in both experiments (*ef*), presumably by unmasking background acid loading that is usually balanced by Na-H exchange at the steady-state pH_i.

Based on segment-*de* pH_i recovery data, such as those in Fig. 4*A*, we compared the pH_i dependence of φ_E after a "2-line/Part EtOH wash" with the pH_i dependence of φ_E after either a "Full EtOH wash" (Fig. 4*B*) or a "Minimal wash" (Fig. 5*A*). In all cases, φ_E decreases at progressively higher pH_i values, but the "2-line/Part EtOH wash" data are slightly alkaline shifted compared to the single-perfusion-line data.

Based on segment-*ef* pH_i decrease data (Fig. 4*A*), we compared the pH_i dependence of φ_{EIPA} after a "2-line/Part EtOH wash" with the pH_i dependence of φ_{EIPA} after either a "Full EtOH wash" (Fig. 4*C*) or a "Minimal wash" (Fig. 5*B*). In all cases, φ_{EIPA} increases at progressively higher pH_i values, but the "2-line/Part EtOH wash" data are markedly alkaline shifted (0.2 – 0.3 pH units) compared to the single-perfusion-line data.

Finally, in Figs. 4*D* and 5*C*, we plot the pH_i dependence of Na-H exchange ($\varphi_{Na\text{-}H}$) for the three wash conditions. The $\varphi_{Na\text{-}H}$ plots are the result of adding the φ_E data (Figs. 4*B* and 5*A*) and the φ_{EIPA} data (Figs. 4*C* & 5*B*) from comparable protocols. In all cases, $\varphi_{Na\text{-}H}$ decreases linearly with increasing values of pH_i, and the pairs of plots in Figs. 4*D* and 5*C* are virtually identical.

The results presented in Figs. 4 and 5 are consistent with the hypothesis that nigericin contamination in the single-line wash protocols (compared to the "2-line/Part EtOH wash" protocol) increases background acid load-

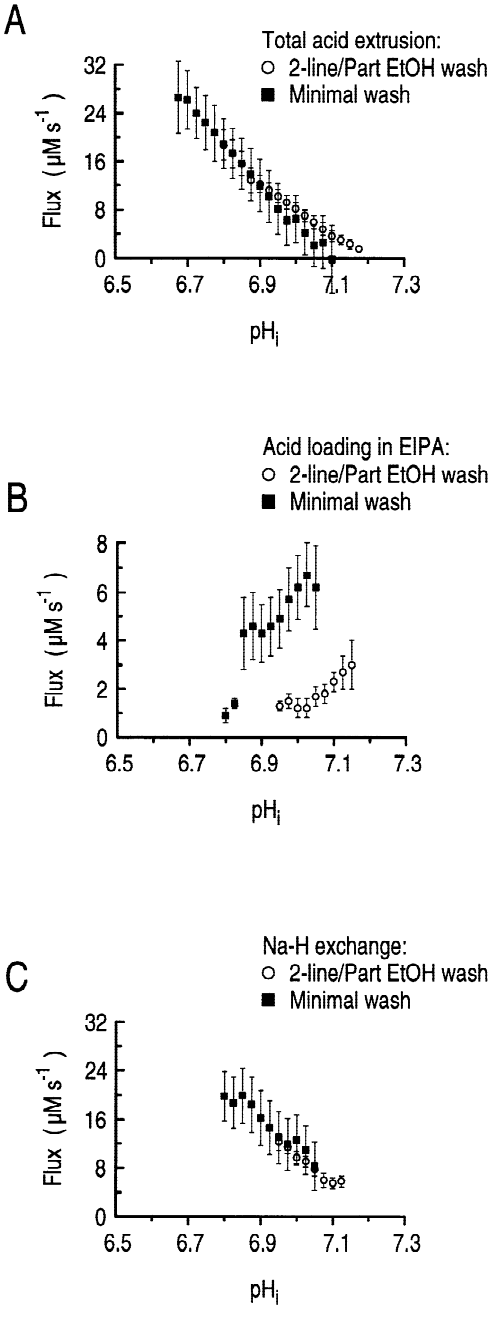


Fig. 5. Contaminating nigericin in "Minimal wash" experiments increases background acid loading of mesangial cells, but has no effect on the pH_i dependence of Na-H exchange. (*A*) The pH_i dependence of total acid extrusion (φ_E) in "Minimal wash" experiments (closed squares) and "2-line/Part EtOH wash" experiments (open circles). φ_E is the product of the rate of the pH_i recovery from an acid load (e.g., segments *de* in Fig. 4*A*) and the pH_i dependence of β_I (*see* legend, Fig. 2*B*). For *A–C*, each point represents an *n* of three or more. The "2-line/Part EtOH wash" data are replotted from Fig. 4*B*. (*B*) The pH_i dependence of background acid loading (φ_{EIPA}) in "Minimal wash" experiments (closed squares) and "2-line/Part EtOH wash" experiments (open circles). φ_{EIPA} is the product of the rate of the pH_i decrease elicited by EIPA (e.g., segments *ef* in Fig. 4*A*) and the pH_i dependence of β_I. The data in *B* represent the same wash protocols as in *A*. The "2-line/Part EtOH wash" data are replotted from Fig. 4*C*. (*C*) The pH_i dependence of Na-H exchange ($\varphi_{Na\text{-}H}$) in "Minimal wash" experiments (closed squares) and "2-line/Part EtOH wash" experiments (open circles). The "2-line/Part EtOH wash" data are replotted from Fig. 4*D*.

ing (Figs. 4*C* and 5*B*), presumably by promoting exchange of internal K^+ for external H^+. As a result, φ_E is slightly lower in the single-line wash protocols (Figs. 4*B* and 5*A*), as revealed by a slight acid shift in the φ_E *vs.* pH_i plots. On the other hand, the residual contaminating nigericin after a single-line wash protocol has virtually no effect on the pH_i dependence of Na-H exchange (Figs. 4*D* and 5*C*).

Discussion

General

In experiments on mesangial cells, we confirm the observation of Richmond and Vaughan-Jones [17] that residual nigericin from the dye calibration in one experiment can interfere with pH_i changes in the next experiment. Because these authors could only minimize nigericin contamination by employing an arduous—and impractical—washing procedure, their group has opted for calibrating intracellular pH-sensitive dyes only about once a month [13, 19]. However, we have found that we can minimize nigericin contamination by using a far more convenient—and practical—approach: Use a separate, dedicated line to deliver nigericin-containing solutions to the cells, and wash the line with ethanol and water after each experiment. As a result, it is feasible to calibrate the dye after each experiment, which should optimize the conversion of optical data to pH_i values, without major effects on acid-base transport. Moreover, we found that even after minimal washing procedures, nigericin contamination does not affect the calculated pH_i dependence of Na-H exchange, provided that one computes this parameter properly. However, when specifically assaying for K-H exchange, one must be especially careful, as were Richmond and Vaughan-Jones [17], not to confuse a physiological K-H exchanger with the ionophore effects of nigericin.

Source of Nigericin Contamination

In attempting to minimize nigericin contamination by excessively washing the perfusion lines with Decon®, we discovered that we could maximally reduce nigericin contamination only if we replaced the perfusion tubing prior to the Decon washing ("Decon® Plus wash"). We therefore suspected that most of the nigericin contamination originates from the Saran® or Tygon® tubing exposed to the nigericin, as opposed to the chamber. Indeed, the chamber has a much smaller surface area than the tubing. When we used a dedicated line to deliver nigericin to the cuvette ("2-line/Part EtOH wash"), so the nigericin solution bypassed the solenoid valves, manifold and most of the tubing, we found the acid-loading rate to be as low as the rate in the "Decon® Plus wash" experiments. The use of a dedicated nigericin delivery line downstream of the manifold minimizes contamination by (i) reducing the amount of perfusion tubing exposed to the ionophore, and (ii) eliminating the possibility that nigericin might diffuse retrograde up the manifold inlet ports, which carries nominally nigericin-free solutions.

Apparently, when one begins an experiment with new tubing, nigericin in the calibration solutions gradually binds to the surfaces of the system. The binding likely increases with the area of surface exposed, as well as with the concentration of nigericin and duration of the exposure—up to a point. During the subsequent experiment, the nigericin evidently dissociates from bound surfaces, and is carried at some finite concentration in the solutions to which the cells are exposed. The extent to which nigericin contamination produces a noticeable effect depends on several factors, including: (i) the amount of nigericin previously bound to the perfusion system, (ii) the ease with which the nigericin dissociates from these surfaces, (iii) the rate of solution flow through the system, (iv) the speed with which nigericin can dissociate from cells, and (v) the sensitivity of the cells to nigericin. In principle, flowing a nigericin-free solution through the perfusion system for a sufficiently long time should eventually deplete the system of bound nigericin. However, we found that even extensive washings (i.e., "Full EtOH wash," "Full Alb wash," and "Decon® wash") can not completely eliminate residual nigericin. Therefore, it is likely that either certain types of tubing are capable of reversibly binding large amounts of nigericin, or that even minute amounts of soluble nigericin are sufficient to produce detectable effects on cells.

Although we investigated nigericin contamination in only one specific solution-delivery system, our observations are likely to be relevant to a large fraction of investigators using nigericin. Our system was constructed of materials that are in widespread use: Stainless-steel tubing, Saran® or Tygon® tubing, solenoid valves, and a Plexiglass® manifold. The assays presented in this study will also allow investigators to determine the extent of nigericin contamination in perfusion systems constructed of other materials. We caution that the specific procedures required to decontaminate a system of nigericin adequately may differ somewhat depending on the nature of the perfusion system, as well as the cell type under investigation. Moreover, it may be difficult to be certain when decontamination is complete if the cell being studied has a native K-H exchanger or, as in the case of our cells, a Na-H exchanger. On the other hand, if one is studying a transporter for which a specific inhibitor is available, it is possible to compute transport activity independent of possible effects of nigericin.

Effect of Nigericin on Background Acid Loading

For cells bathed in the standard HEPES-buffered solution, nigericin should exchange internal K^+ for external

H^+, and thus contribute to the intracellular background acid loading of the cells. Not surprisingly, we found that the plot of total acid extrusion *vs.* pH_i extrapolated to a pH_i of ~7.1 for cells subjected to a "Minimal wash," but ~7.2 for cells subjected to the more rigorous "2-line/Part EtOH wash." Thus, it is likely that nigericin contamination can cause a slight acid shift in steady-state pH_i.

In experiments on type-1 cells of neonatal rat carotid body, Richmond and Vaughan-Jones [17] concluded that nigericin contamination increased "background acid loading," as measured by an increase in the rate of acid loading elicited by removing extracellular Na^+ and simultaneously adding amiloride to the extracellular fluid ("SFA" solution). However, as we have pointed out in the present study, removing external Na^+ will likely elicit a pH_i decrease due to the introduction of a nigericin-mediated exchange of internal Na^+ or K^+ for external H^+. Therefore, in the carotid-body study, the pH_i decrease elicited by exposing the cells to the "SFA" solution probably represents—at least in part—*stimulation* of nigericin-mediated exchange of internal Na^+ for external H^+, not simply "background acid loading" as commonly defined [7].

In our experiments on mesangial cells, we determined the pH_i dependence of background acid loading under different wash protocols by inhibiting the Na-H exchanger with EIPA. As shown in Figs. 4*C* and 5*B*, compared to the plot of the pH_i dependence of φ_{EIPA} after a "2-line/Part EtOH wash," the plots for a "Minimal wash" and "Full EtOH wash" are acid-shifted. Contaminating nigericin also slightly acid shifts the plots of φ_E *vs.* pH_i (Figs. 4*B* and 5*A*), but has no effect on the pH_i dependence of $\varphi_{Na\text{-}H}$ (Figs. 4*D* and 5*C*). This lack of effect of contaminating nigericin on the computed pH_i dependence of $\varphi_{Na\text{-}H}$ is not surprising because the effects of the ionophore on φ_E and φ_{EIPA} cancel each other out. This exercise demonstrates how misleading it is to regard the gross pH_i-recovery rate (i.e., Figs. 4*B* and 5*A*) as an index of the activity of an acid extruder. The data derived only from the gross pH_i-recovery rate include not only the acid extruder, but also background acid loading, which can be increased by contaminating nigericin.

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