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# The $Na^+/H^+$ antiporter in rat alveolar type II cells and its role in stimulated surfactant secretion

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**We used the pH-sensitive fluorescent probe 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to identify $Na^+/H^+$ exchange in freshly isolated rat alveolar type II cells and alveolar type II cells in primary culture. The intracellular pH ($pH_i$) of freshly isolated alveolar type II cells was 7.36 ± 0.05 ($n = 3$). When freshly isolated alveolar type II cells were acid loaded with nigericin in sodium-free buffer, the $pH_i$ dropped to 6.59 ± 0.04 and remained low in sodium-free buffer. When acid-loaded cells were subsequently incubated with NaCl, $pH_i$ increased in a dose-dependent manner. Amiloride (0.1 mM) inhibited the sodium-induced increase in $pH_i$. When the acid-loaded cells were resuspended in an unbuffered choline chloride solution, the cells secreted $H^+$ in a sodium-dependent and amiloride-inhibitable manner. Alveolar type II cell monolayers, which were cultured for 22 h on glass coverslips and then loaded with BCECF, had a resting $pH_i$ of 7.48 ± 0.05 ($n = 4$). Nigericin acidified these cultured cells in the absence of sodium and NaCl increased the $pH_i$ of these acid loaded cells as observed in freshly isolated cells. Secretagogues of pulmonary surfactant, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and terbutaline, did not change $pH_i$. Inhibition of the $Na^+/H^+$ antiporter by the addition of amiloride to a $Na^+$ containing medium or the substitution of choline for $Na^+$ did not inhibit stimulated phosphatidylcholine secretion. We conclude that $pH_i$ regulation in rat alveolar type II cells is in part mediated by an amiloride-sensitive $Na^+/H^+$ antiporter, but this system appears not to be involved in TPA- or terbutaline-induced pulmonary surfactant secretion in primary culture.**

## Introduction

The existence of a $Na^+/H^+$ exchange system has been documented in the plasma membrane of a wide variety of animal cells [1]. In epithelial cells where this proton exchange system has been identified, it may play a role in the specialized function of transepithelial ion and solute transport. As an example, in the renal proximal tubule epithelium the $Na^+/H^+$ antiporter appears to play a major role in $H^+$ secretion and transepithelial $Na^+$ transport, and can be inhibited by the diuretic amiloride [2]. More recently an analogous $Na^+/H^+$ antiporter has been detected in several nonpolar cells [3–9] including fibroblasts [6], lymphocytes [7], neutrophils [8] and platelets [9]. In nonepithelial cells this antiporter has been reported to be involved in a number of important

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein.

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cell functions, including volume regulation [10], the control of intracellular pH ($pH_i$) [7], mitogenesis [6], and the oxidative burst of neutrophils [8].

The presence of a $Na^+/H^+$ antiporter in alveolar epithelial cells, such as alveolar type II cells, was initially suspected because of indirect physiologic evidence. Alveolar type II cells are located in the corners of alveoli and are responsible for the synthesis and secretion of pulmonary surfactant into the alveolar lining layer. The alveolar lining layer consists of two parts: a monomolecular film of surface active material at the air/liquid interface and the aqueous subphase. The pH of the aqueous subphase is acidic (pH 6.92 for rabbits [11] and pH 6.27 for fetal lambs [12]). An active $H^+$ (or $HCO_3^-$) transport by the alveolar epithelium has been postulated as a mechanism that may contribute to the acidification of the aqueous subphase [11]. Alveolar type II cells actively transport sodium from their apical to basolateral surface [13] and $Na^+/K^+$-ATPase has been localized to their basolateral membrane [14]. The $Na^+/H^+$ antiporter has been localized to the apical membrane of renal epithelial cells capable of active ion transport [15,16]. Therefore, a $Na^+/H^+$ antiporter similarly located in alveolar type II cells may play a major role in the maintenance of an acidic aqueous subphase.

Recently, the presence of a $Na^+/H^+$ exchange pathway has been confirmed in suspensions of freshly isolated rat alveolar type II cells [17]. However, little is known about the role of this antiporter in the cellular functions of the alveolar type II cell. Alveolar type II cells are unique epithelial cells containing large secretory granules called lamellar bodies whose contents are secreted to form alveolar surface active material [18]. Among various agents tested, the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) is a very potent stimulant of surfactant secretion from alveolar type II cells in primary culture [19]. TPA also stimulates the $Na^+/H^+$ antiporter in many cell systems [8,20–23]. The purpose of the present work was to determine if a $Na^+/H^+$ antiporter is present in both freshly isolated and primary cultures of alveolar type II cells and whether this antiporter plays a role in TPA- or terbutaline-induced surfactant secretion.

## Materials and Methods

*Animals and materials.* Pathogen-free Sprague-Dawley rats (weighing 200–250 g) were obtained from Bantin-Kingman, Inc. (Freemont, CA). The sources of materials used in this work were as follows: 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein tetraacetoxymethyl ester (BCECF/AM) from Molecular Probes Inc. (Junction City, OR); elastase (porcine pancreas) from Cooper Biomedical (Malvern, PA); 12-*O*-tetradecanoylphorbol 13-acetate (TPA) from Consolidated Midland Corporation (Brewster, NY); terbutaline sulfate from Merrell Dow Pharmaceuticals Inc. (Cincinnati, OH); [*N-Me*-$^3$H]choline chloride (80 Ci/mmol) from New England Nuclear (Boston, MA); amiloride from Merck Sharp and Dohme (West Point, PA); nigericin from Calbiochem. (San Diego, CA); *N*-methyl-D-glutamine (NMG) from Sigma (St. Louis, MO).

*Isolation of alveolar type II cells.* Alveolar type II cells were isolated from adult male Sprague-Dawley rats by tissue dissociation with elastase and partial purification on metrizamide density gradients [24]. The cells were further purified by centrifugal elutriation as described previously [25] for experiments using freshly isolated cells or by differential adherence in primary culture for 22 h. After elutriation the cell purity determined by a modified Papanicolaou stain [26] was 92 ± 2% and the cell viability determined by Trypan blue exclusion was 95 + 3% (mean ± S.D., $n = 6$). The cell purity after 22 h culture was 95 ± 3% and viability was 96 ± 2% (mean ± S.D., $n = 14$).

*Measurement of $pH_i$ in elutriated alveolar type II cells.* The $pH_i$ in freshly isolated alveolar type II cells, which were purified by elutriation, was measured by using the pH-sensitive fluorescent dye BCECF [27]. The following solutions were used: NaCl-Hepes buffer (145 mM NaCl, 10 mM Hepes-K, 5 mM KCl, 0.5 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5.5 mM glucose (pH 7.4)); NMG-Hepes buffer (identical to NaCl-Hepes buffer except that 145 mM *N*-methyl-D-glucamine was substituted for NaCl (pH 7.4)); KCl-Hepes buffer (107 mM KCl, 51 mM NaCl, 10 mM Hepes-K, 0.5 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5.5 mM glucose (pH 7.4)); and choline chloride solution (145 mM choline chloride, 5 mM KCl, 0.5 mM $CaCl_2$, 0.8

mM $MgCl_2$ (pH 7.4, adjusted with 1 M KOH)). The concentrations of $Na^+$ and $K^+$ in the KCl-Hepes buffer are the same as the reported intracellular concentrations of these ions in rat alveolar type II cells [28]. Elutriated alveolar type II cells ($10^8$ cells/ml) were incubated with 3 $\mu$M BCECF/AM (which is hydrolyzed to BCECF by intracellular esterases) in NaCl-Hepes buffer for 1 h at room temperature. After BCECF loading, the cells were diluted to a concentration of $4 \cdot 10^6$ cells/ml with NaCl-Hepes buffer, centrifuged at 1000 rpm ($260 \times g_{max}$) for 10 min, and then resuspended in NaCl-Hepes buffer, NMG-Hepes buffer, or KCl-Hepes buffer as indicated at $40 \cdot 10^6$ cells/ml. Aliquots of cells were then transfered to quartz cuvettes (Fisher, Pittsburg, PA.) in the appropriate buffer to final volumes of 2 ml with $1 \cdot 10^6$ cells/ml. The cuvettes were loaded in a water-jacketed holder maintained at 37°C, cell suspensions were stirred with an automatic stirring device (Instech Laboratories, Horsham, PA), and fluorescence intensity was determined using a Perkin-Elmer 650-10S fluorescence spectrophotometer (Norwalk, CN) equipped with a recorder (Pharmacia, Piscataway, NJ). In preliminary experiments peak fluorescence intensity of BCECF in alveolar type II cells was found to be an excitation wavelength of 506 nm and an emission wavelength of 526 nm. Therefore, these wavelength settings were used in all subsequent studies of BCECF-loaded alveolar type II cells. The leakage of BCECF during experiments was determined by measuring the fluorescence of supernatants of the cell suspension and was less than 5% over 10 min.

In some experiments, cells were acid-loaded with nigericin as follows: The BCECF-loaded cells were suspended in NMG-Hepes buffer ($(20–40) \cdot 10^6$ cells/ml) and then incubated with 1.0 $\mu$g/ml nigericin for 7 min at room temperature. Bovine serum albumin (4 mg/ml) was added to scavenge the ionophore. The cells were then centrifuged and resuspended in NMG-Hepes buffer at a concentration of $10^8$ cells/ml. Aliquots of this cell suspension were then added to the appropriate buffer in the cuvettes to make final volumes of 2 ml at $1 \cdot 10^6$ cells/ml.

*Measurement of $H^+$ secretion by elutriated alveolar type II cells.* Elutriated alveolar type II cells were resuspended in NMG-Hepes buffer and were then acid loaded with 1.0 $\mu$g/ml of nigericin as described above. The cells were washed and resuspended in choline chloride solution ($1 \cdot 10^6$ cells/ml). The cells suspension (2 ml) was gently stirred and the pH of the solution ($pH_o$) was measured using a microcombination pH probe (Microelectrodes, Inc. model M1-410, Londonderry, NH) attached to a pH meter (Orion ionanalyzer model EA920, Cambridge, MA) and equipped with a recorder (Pharmacia). These experiments were conducted at room temperature.

*Meausrements of $pH_i$ in alveolar type II cells in primary culture.* Cells recovered from metrizamide density gradients were suspended at $1 \cdot 10^6$ cells/ml in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 $\mu$g/ml gentamicin, 100 U/ml penicillin, and 50 $\mu$g/ml streptomycin. The cells were plated at $5 \cdot 10^5$ cells/well in a 24-well plate which contained a 13-mm glass coverslip in each well. The coverslips had been acid washed with a chromic sulfuric acid solution (Chromerge, Fisher), multiply rinsed with distilled water, and then alcohol-flame sterilized prior to being placed in the 24-well plate. After incubation in an atmosphere of 10% $CO_2$/90% air for 22 h at 37°C, the monolayers were washed with NaCl Hepes buffer and then loaded with BCECF/AM (3 $\mu$g/ml) in NaCl-Hepes buffer for 1–3 h at room temperature just preceding the experimental period. Fluorescence microscopy of BCECF-loaded alveolar type II cell monolayers showed the dye to be very evenly distributed throughout the cytoplasm of the cells. Fluorescence was not observed in the lamellar bodies probably because of the acidic nature of these organelles [29]. After BCECF loading, the coverslips with monolayers were washed in the buffer to be used in the assay and then inserted in 3-ml cuvettes containing 3 ml of the same buffer. The cuvette was placed in a water-jacketed cuvette holder maintained at 37°C and oriented in the flourimeter such that the incident light struck the cell monolayer at a 45° angle. The monolayer on its coverslip remained in a fixed position within the cuvette throughout the experiment. The autofluorescence of alveolar type II cell monolayers was approximately 4% of that of the BCECF-loaded monolayers.

*Secretion of phosphatidylcholine from alveolar*

*type II cells.* Cells recovered from the metrizamide density gradient were suspended at $1 \cdot 10^6$ cells/ml in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 μg/ml gentamicin, 100 U/ml penicillin, 50 μg/ml streptomycin, and 1 μCi/ml [*N-Me*-$^3$H]choline chloride. The cells were plated at $2 \cdot 10^6$ cells/35 mm dish. After incubation for 22 h in 10% $CO_2$/90% air at 37°C, the dishes were washed with 10 ml of DMEM containing 1 mg/ml bovine serum albumin to remove non-adherent cells and radioactive materials. In the amiloride and $Na^+$ substitution experiments, an additional wash was performed with either NaCl-Hepes buffer or choline chloride solution as indicated. In the BCECF experiments, the cells were preincubated for 1 h at room temperature in NaCl-Hepes buffer with or without BCECF/AM (3 μg/ml) before the addition of stimulants. The appropriate experimental medium and stimulants were then added and the incubation was continued for 1 h at 37°C (in an air atmosphere for media not containing bicarbonate buffer). After the 1 h period of stimulation, the medium was removed, centrifuged at 1000 rpm ($260 \times g_{max}$) for 10 min to remove any detached cells, and the lipid was extracted by the method of Bligh and Dyer [30]. The radioactivity in the total lipid fraction was determined as previously described [31]. Under these conditions, 98.4% of the radioactivity in the total lipid fraction is in phosphatidylcholine [31]. The cells were harvested from dishes with a rubber policeman and treated in the same manner as the media. The percentage release of phosphatidylcholine was calculated as follows: % secretion = (total lipid cpm in medium/total lipid cpm in cells + total lipid cpm in medium) × 100. For each experimental condition, medium and harvested cells were assayed for lactate dehydrogenase (LDH), a cytoplasmic enzyme, and the percent of lactate dehydrogenase released was determined in order to quantitate cytotoxicity [32].

## Results

### *$pH_i$ in elutriated alveolar type II cells*

We calibrated fluorescence intensity of BCECF in terms of $pH_i$ by two methods as shown in Fig. 1. The freshly isolated and elutriated, alveolar

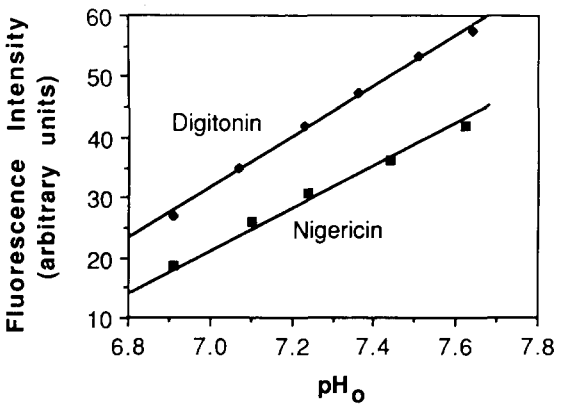


Fig. 1. Comparison of nigericin (intracellular, ■) vs. digitonin (extracellular, ◆) calibration in elutriated alveolar type II cells. Alveolar type II cells were purified by elutriation, loaded with BCECF, and resuspended in KCl-Hepes solution. Nigericin (2 μg/ml) was added and $pH_o$ was titrated with either 1 M NaOH or 1 M HCl and fluorescence was measured at each $pH_o$ value. Digitonin (20 μg/ml) was then added and a second titration was performed. The fluorescence intensity at different $pH_o$ values of nigericin-treated vs. digitonin-treated cells was compared. These data are representative of four different experiments with similar findings.

type II cells were loaded with BCECF and then resuspended in KCl-Hepes buffer and 2 μg/ml nigericin was added to equilibrate $pH_i$ and $pH_o$. The $pH_o$ was titrated with 1 M HCl or 1 M NaOH across a range of fluorescence readings to obtain a fluorescence vs. pH calibration curve. 20 μg/ml of digitonin was then added to release the dye into free solution and another calibration curve was constructed. As shown, at any given value of $pH_o$ the fluorescence from the intracellular dye (nigericin-high $K^+$) was suppressed compared with the external dye (digitonin). As a consequence, the digitonin-derived $pH_i$ values underestimated the actual $pH_i$ values. Similar comparative curves were obtained for elutriated and BCECF-loaded alveolar type II cells from each cell preparation ($n = 4$ cell preparations). This result is consistent with the observations made by other investigators using different cell systems [27,33]. To avoid additional manipulation of the cells (centrifugation, washing, and resuspension), all fluorescence measurements not utilizing the KCl-Hepes buffer were followed by digitonin (extracellular) calibration and the pH obtained in this manner was corrected upward according to the two calibration curves which were obtained for each experiment. Thus,

all pH values are expressed in terms of an intracellular (nigericin-high $K^+$) calibration.

The resting $pH_i$ of elutriated alveolar type II cells was found to be $7.36 \pm 0.05$ (mean $\pm$ S.E., $n = 3$ different cell preparations). When the cells were acid loaded with 1.0 $\mu$g/ml of nigericin in NMG-Hepes buffer, $pH_i$ dropped to $6.59 \pm 0.04$ (mean $\pm$ S.E., $n = 3$ different cell preparations) and remained low in the NMG-Hepes buffer as shown in Fig. 2A. When acid-loaded cells were resuspended in Hepes-buffered solution containing various concentrations of NaCl, $pH_i$ increased in a NaCl concentration-dependent manner and amiloride (0.1 mM) inhibited the NaCl-induced cellular alkalinization (Fig. 2B). The shape of Fig. 2B suggests saturable carrier mediated transport. Assuming a constant intracellular buffering capacity over the range of $\Delta pH_i$ and using a Woolf-Hofstee plot [34,35], the mean data from Fig. 2B can be plotted as a straight line ($r = 0.99$ by linear regression analysis) and an apparent $K_m$ for $Na^+$ of 18 mM can be derived. A Hill plot [34–36] results in a straight line approximation ($r = 0.99$

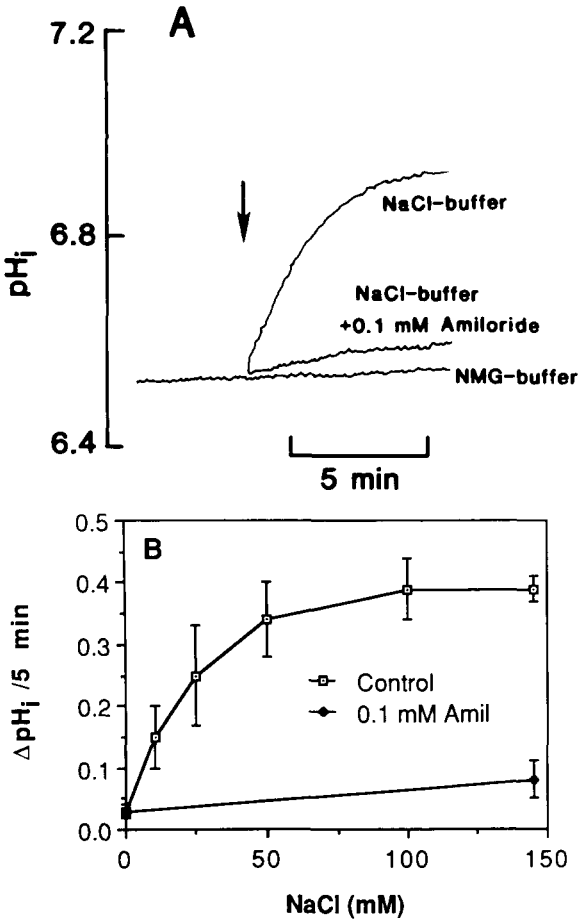


Fig. 2. External $Na^+$ dependence of $Na^+/H^+$ exchange. (A) Representative fluorescence traces of the cytoplasmic alkalinization elicited by external $Na^+$. Elutriated alveolar type II cells were loaded with 3 $\mu$M BCECF and then acidified by nigericin (1 $\mu$g/ml) in NMG-Hepes buffer for 7 min. Nigericin was scavenged with 4 mg/ml bovine serum albumin and then the cells were centrifuged. The acidified cells were resuspended in NMG-Hepes buffer, NaCl-Hepes buffer, or NaCl-Hepes buffer containing 0.1 mM amiloride. (B) Dependence of cytoplasmic alkalinization on extracellular $Na^+$. The acidified cells were resuspended in the Hepes-buffered solution containing various concentrations of NaCl (□) or NaCl plus 0.1 mM amiloride (◆) and the change in $pH_i$ was recorded. The osmolarity in the buffer was adjusted with *N*-methyl-D-glucamine. Each point represents the mean $\pm$ S.E. of three different experiments.

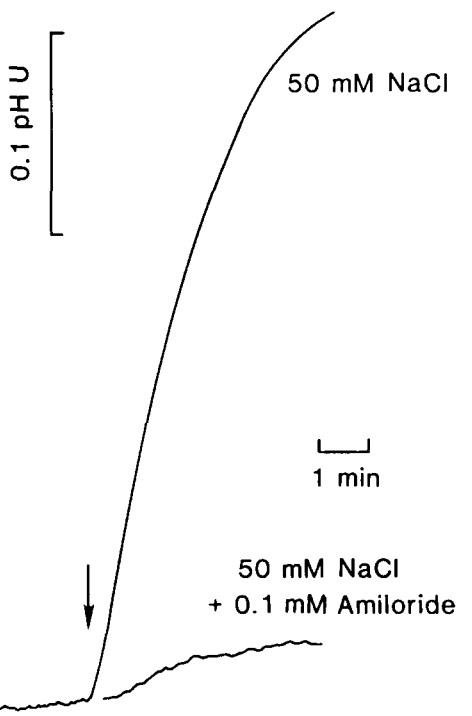


Fig. 3. $Na^+$-induced extracellular acidification. Elutriated alveolar type II cells were acidified with nigericin (1 $\mu$g/ml) in NMG-Hepes buffer for 7 min. Nigericin was scavenged with 4 mg/ml bovine serum albumin and the cells were centrifuged. The acidified cells were resuspended in choline chloride solution. NaCl with or without 0.1 mM amiloride was added at the time indicated by the arrow from a 5 M NaCl stock solution to give a final concentration of 50 mM. The external pH was continuously recorded with an upward displacement of the curve indicating increased acidification. The data are representative of three different experiments with similar findings.

by linear regression analysis) with a slope or Hill coefficient of 0.96. This value is approximately one, indicating an apparent lack of cooperativity of the antiporter [35] and a probable 1 : 1 $Na^+/H^+$ exchange ratio [1,35].

*$Na^+$-induced $H^+$ efflux from elutriated alveolar type II cells*

To confirm that NaCl-induced cellular alkalinization was dependent upon a $Na^+/H^+$ exchange process, we measured the change of $pH_o$. Elutriated alveolar type II cells were acid-loaded with nigericin in NMG-Hepes buffer and then resuspended in choline chloride solution. This solution was buffered only by any carried-over Hepes from the pelleted cells. The $pH_o$ showed only minimal changes under this condition but subsequent addition of 50 mM NaCl rapidly acidified the extracellular solution (Fig. 3). This NaCl-induced $H^+$ efflux was inhibited by 0.1 mM amiloride. The addition of the same concentration of choline chloride did not change $pH_o$ (data not shown).

*$pH_i$ of alveolar type II cells in primary culture*

Alveolar type II cells in primary culture attach to a substratum and form a polarized monolayer from which phosphatidylcholine, the major phospholipid component of surfactant, is secreted [19,37]. We were interested in determining whether TPA- or terbutaline-stimulated phosphatidylcholine secretion was associated with activation of a $Na^+/H^+$ antiporter and cellular alkalinization. Therefore, we initially conducted experiments to assure that the $Na^+/H^+$ antiporter was retained in alveolar type II cells in primary culture.

To calibrate BCECF fluorescence in terms of $pH_i$ for alveolar type II cell monolayers, we adapted the technique that Selvaggio et al. [38] used in calibrating BCECF fluorescence in MDCK cell monolayers. Initially, we determined that the fluorescence intensity of BCECF-loaded alveolar type II cell monolayers at an excitation of 455 nm (emission 526 nm) was relatively insensitive to pH changes. In addition, the ratio of fluorescence intensities at an emission wavelength of 526 nm and excitations of 506 nm (excitation wavelength for peak fluorescence intensity) and 455 nm was proportional to $pH_o$ in the presence of 2 $\mu$g/ml nigericin in KCl-Hepes buffer (Fig. 4). This tech-

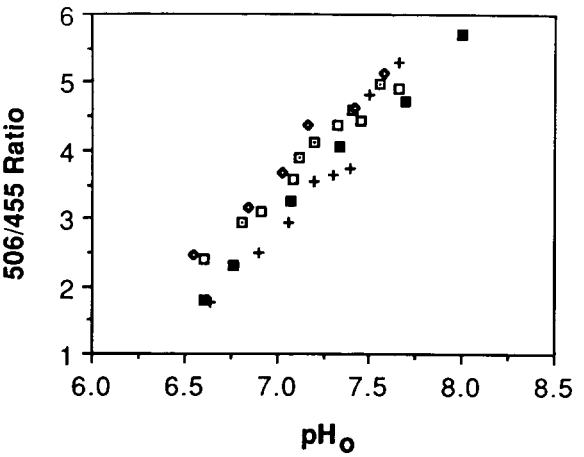


Fig. 4. Relationship between fluorescence intensity ratio of BCECF (505/455 ratio) and intracellular pH. BCECF loaded alveolar type II cell monolayers on glass coverslips were inserted in cuvettes containing 3 ml of KCl-Hepes buffer and nigericin (2 $\mu$g/ml) was added. The extracellular pH ($pH_o$) was titrated with either 1 M HCl or 1 M NaOH to obtain various $pH_o$ values. Excitation wavelength was scanned and the 506/455 ratio (as described under Results) was calculated at each different $pH_o$. The data represent five different experiments (shown as different symbols) with similar findings.

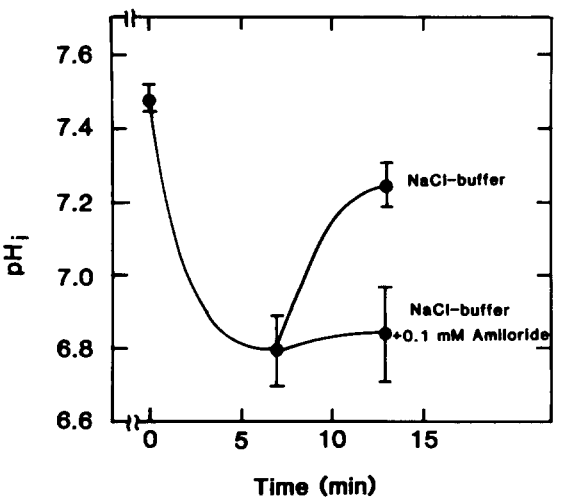


Fig. 5. $Na^+$-dependent cellular alkalinization of acidified type II cell monolayers. BCECF loaded alveolar type II cell monolayers on glass coverslips were acidified with nigericin (1 $\mu$g/ml) in NMG-Hepes buffer for 7 min. Then the buffer was changed to NaCl-Hepes buffer with or without 0.1 mM amiloride and the 506/455 ratio was measured after 5 min. Each point represents the mean ± S.E. of four different experiments. The lines between the points are approximated from the fluorescent tracings.

nique also proved to be very reproducible from experiment to experiment as shown in Fig. 4. These observations are similar to those made by Selvaggio et al. [38] using MDCK monolayers and allow for the calibration of BCECF fluorescence intensity in terms of $pH_i$ by using the ratio of fluorescence intensity at an excitation of 506 nm to the fluorescence intensity at an excitation of 455 nm (506/455 ratio). Using this calibration method the $pH_i$ of alveolar type II cell monolayers in NaCl-Hepes buffer was determined to be $7.48 \pm 0.05$ (mean $\pm$ S.E., $n = 4$ different cell preparations). Nigericin (1.0 $\mu$g/ml) acidified the cells in NMG-Hepes buffer to a pH of $6.79 \pm 0.09$ in 7 min (mean $\pm$ S.E., $n = 4$ different cell preparations) as shown in Fig. 5. In addition, a NaCl-induced cellular alkalinization was observed for acid loaded alveolar type II cells in primary culture that was inhibited by amiloride (Fig. 5).

*Effects of stimulants of surfactant secretion on $pH_i$*

We confirmed the previous observations [19,37] that both TPA and terbutaline stimulate phosphatidylcholine secretion from alveolar type II cells in primary culture (Tables I and II). Loading cells with BCECF did not affect the basal or stimulated secretion (Table I). In addition, there was no significant difference in lactate dehydrogenase release between BCECF-loaded cells and control cells. The percent of lactate dehydrogenase release did not exceed 3% of total cellular lactate dehydrogenase activity for any of the experimental or control conditions. Therefore, BCECF did not appear to adversely affect alveolar type II cell phosphatidylcholine secretion.

TABLE I

EFFECT OF BCECF ON PHOSPHATIDYLCHOLINE SECRETION

Freshly isolated alveolar type II cells were incubated for 22 h in medium containing 1 $\mu$Ci/ml of [$^3$H]choline chloride. The cells were then washed and incubated in NaCl-Hepes buffer with or without 3 $\mu$M BCECF for 1 h. Saline, TPA (50 ng/ml), or terbutaline (100 $\mu$M) was then added and the incubation was continued for an additional 1 h at 37 °C. The % secretion was then determined as described under Materials and Methods. The data are expressed as mean $\pm$ S.E. from three different experiments ($n = 3$) and with duplicate samples being averaged for each condition in each experiment. For each addition, there was no significant difference between values obtained either with (+BCECF) or without (−BCECF) BCECF.

| Addition | % Secretion | |
|---|---|---|
| | −BCECF | +BCECF |
| Saline | 2.6 ± 1.2 | 2.8 ± 1.3 |
| Terbutaline | 7.5 ± 0.2 | 6.5 ± 0.4 |
| TPA | 20.2 ± 1.3 | 19.6 ± 1.2 |

We then examined the effect of TPA and terbutaline on $pH_i$ in alveolar type II cells in primary culture. The BCECF-loaded alveolar type II cell monolayers on glass coverslips were washed in saline and then inserted into cuvettes containing 3 ml of NaCl-Hepes buffer. Saline, TPA (50 ng/ml), or terbutaline (100 $\mu$M) was then added and the fluorescence was continuously recorded. The 506/455 ratio was determined at 0 min, 5 min, 15 min, and 30 min after the addition. Although TPA stimulates a $Na^+/H^+$ antiporter in several other cell systems, we found that neither TPA nor terbutaline changed $pH_i$ in alveolar type II cell monolayers. For example, the $pH_i$ at 15 min after the addition of saline or secretagogues was: $7.45 \pm 0.05$ for the control, $7.44 \pm 0.04$ with TPA, and $7.46 \pm 0.04$ with terbutaline (mean $\pm$ S.E., $n = 4$ different cell preparations). In addition, there was no change in $pH_i$ (alkalinization or acidification) after stimulants were added to cuvettes containing monolayers bathed by NaCl-Hepes buffer plus $10^{-3}$ M amiloride. This observation makes it unlikely that stimulants cause co-existent intracellular proton production and compensatory $Na^+/H^+$ antiporter activation to maintain a constant intracellular pH. Finally, neither TPA nor terbutaline changed $pH_i$ in freshly isolated-elutriated alveolar type II cell suspensions (data not shown).

To determine the dependency of phosphatidylcholine secretion on $Na^+$ transport, secretion studies were performed in the presence and absence of amiloride and in a $Na^+$substituted medium (residual $Na^+ < 3$ mM). Neither terbutaline- nor TPA-stimulated secretion were inhibited by the addition of amiloride to a $Na^+$ containing medium or the medium substitution of choline for $Na^+$ (Table II). Thus, inhibition of $Na^+/H^+$ exchange did not inhibit stimulated phopshatidylcholine secretion. Normal stimulated secretion also took place in a choline chloride solution buffered with choline bicarbonate (data not

TABLE II

EFFECT OF AMILORIDE AND SODIUM SUBSTITUTION ON PHOSPHATIDYLCHOLINE SECRETION

Freshly isolated alveolar type II cells were incubated for 22 h in medium containing 1 $\mu$Ci/ml of [$^3$H]choline chloride. The cells were then washed and incubated for 1 h at 37 °C in the experimental media indicated with or without $10^{-3}$ M amiloride and with the stimulants indicated (control, 50 ng/ml TPA or 100 $\mu$M terbutaline). The percent secretion was then determined as described under Materials and Methods. The data are expressed as mean ± S.E. from four different experiments ($n = 4$) and with duplicate samples being averaged for each condition in each experiment. Terbutaline- and TPA-stimulated secretion were significantly different ($P < 0.05$) from their respective control values for each experimental condition. However, for each addition, there was no significant difference between the values obtained under the different experimental conditions.

| Addition | % Secretion | | |
|---|---|---|---|
| | NaCl-Hepes (− amiloride) | NaCl-Hepes (+ amiloride) | Choline chloride (− amiloride) |
| Control | 1.3 ± 0.2 | 1.2 ± 0.3 | 2.2 ± 0.2 |
| Terbutaline | 5.0 ± 0.9 | 3.9 ± 0.5 | 4.7 ± 0.1 |
| TPA | 9.7 ± 1.1 | 10.2 ± 2.2 | 10.2 ± 0.6 |

shown), indicating that secretion in these experiments was not dependent on the modest cell alkalinization occurring when a $CO_2/HCO_3^-$ containing medium (DMEM) was replaced with a non-$CO_2$/non-$HCO_3^-$ containing medium (NaCl-Hepes or choline chloride solution).

## Discussion

Our data present several lines of evidence to indicate that an amiloride-sensitive $Na^+/H^+$ antiporter exists in rat alveolar type II cells. (1) $Na^+$ induced an alkalinization in cells that were acidified with nigericin (Fig. 2). (2) $Na^+$ induced an extracellular acidification of cell suspensions in which the cells had been previously acidified with nigericin (Fig. 3). (3) Both the $pH_i$ and $pH_o$ changes induced by $Na^+$ were inhibited by amiloride. Taken together, these results suggest the existence of a $Na^+/H^+$ antiporter in the plasma membrane of rat alveolar type II cells and suggest a role for the antiporter in $pH_i$ regulation. Nord et al. [17] have also described a $Na^+$ dependent and amiloride-inhibitable alkalinization of rat alveolar type II cells in suspension. The resting $pH_i$ of cells in their study was 7.07 ± 0.07 and kinetic analysis yielded a $K_m$ for $Na^+$ of 62 ± 3 mM. Differences between the resting pH values obtained in our study and that of Nord et al. may reflect differences in isolation and experimental techniques, and since the rate of antiporter activity and thus the $K_m$ is usually dependent upon the starting $pH_i$, the differences in $K_m$ values are probably due to differences in $pH_i$ values obtained after acidification. Despite these minor differences, both studies demonstrate that a $Na^+/H^+$ antiporter with kinetic properties similar to those of $Na^+/H^+$ antiporters in other cell systems [1] is present in the plasma membrane of alveolar type II cells in suspension.

The $Na^+/H^+$ antiporter has been reported to play a role in a number of important cell functions. Phorbol esters such as TPA can rapidly (seconds to minutes) stimulate $Na^+/H^+$ exchange in a number of cell types through a mechanism that may involve activation of protein kinases [39]. In alveolar type II cells, TPA- and terbutaline-induced phosphatidylcholine secretion appears to be mediated through activation of protein kinase C and a cyclic-AMP dependent protein kinase, respectively [31]. Therefore, we were interested in determining the role of the $Na^+/H^+$ antiporter in the stimulation of surfactant secretion from alveolar type II cells. In initial experiments we confirmed that the $Na^+/H^+$ antiporter was retained in primary cultures of alveolar type II cells and that BCECF loading did not affect basal or stimulated secretion. In preliminary experiments to verify our experimental methods, we also confirmed the observation by Grinstein et al. [23] that TPA alkalinized rat thymocytes (data not shown). However, neither TPA nor terbutaline appeared to stimulate $Na^+/H^+$ exchange in rat alveolar type II cells. Moreover, the stimulation of phosphatidylcholine secretion by either TPA or terbutaline was not dependent on the activation of the $Na^+/H^+$ antiporter. Thus, the $Na^+/H^+$ antiporter does not appear to be involved in the mechanism of TPA- or terbutaline-induced surfactant secretion from primary cultures of rat alveolar type II cells.

Similar observations regarding the absence of a

$Na^+/H^+$ antiporter response have been made in platelets where phorbol esters activate protein kinase C and cause platelet aggregation and serotonin release [40]. However, like rat alveolar type II cells, stimulation of platelets with a phorbol ester has no significant effect on resting $pH_i$ although these cells do contain a $Na^+/H^+$ antiporter [9]. The reason for these variable cell-type dependent effects of phorbol esters on $Na^+/H^+$ antiporter stimulation is unknown, but they may reflect cell-type dependent differences in the $Na^+/H^+$ antiporter or secondarily produced mediators (e.g. from protein kinase activation).

In contrast to our findings, Finkelstein and Brandes [41] in a recent abstract reported that both TPA and terbutaline stimulated a $Na^+$-dependent and amiloride-inhibitable alkalinization of rabbit alveolar type II cells. One possible explanation for these discrepancies is that the cells used in the two studies were obtained from different species *. Effros et al. [42] have reported that electrolyte transport across the pulmonary epithelium may also differ between these two species. Further investigation will be necessary to determine whether other species related differences in the mechanism of surfactant secretion can be identified.

Alveolar type II cells are also capable of transepithelial $Na^+$ transport from their apical to basolateral surface [13] and as such, probably play a major role in maintaining alveolar fluid homeostasis. As in other $Na^+$ transporting epithelial cells, $Na^+/K^+$-ATPase has been localized to the basolateral membrane of alveolar type II cells [14]. In this study we have documented the presence of a $Na^+/H^+$ antiporter in both alveolar type II cells in suspension and in primary cultures of cells which form polarized monolayers. The $Na^+/H^+$ antiporter has been localized to the apical membrane of other epithelial cells capable of active ion transport [15,16]. Thus, a similarly located $Na^+/H^+$ antiporter in alveolar type II cells might play a role in both transepithelial $Na^+$ transport and acidification of the aqueous alveolar subphase. The extent of the $Na^+/H^+$ antiporter's contribution to these processes, however, has yet to be determined.

* See Note added in proof.

**Note added in proof**: (Received 3 March 1988)

Species differences in protein structure and response to inhibitors have recently been identified between rat and rabbit renal $Na^+/H^+$ antiporters [43].

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