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Lateral Mobility of Na,K-ATPase and Membrane Lipids in Renal Cells. Importance of Cytoskeletal Integrity

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Abstract. Because membrane fluidity is an important determinant of membrane function, the lateral diffusion rate (D_L) of the membrane protein Na,K-ATPase was determined in intact renal proximal tubule epithelial cells by the technique of fluorescence redistribution after photobleaching (FRAP). In normal cells the D_L of Na,K-ATPase in the basal membrane was 3.31×10^{-10} cm^2/sec. Treatment with cytochalasin D to promote actin filament depolymerization caused a sevenfold increase in D_L. Exposure of cells to a Ca^{2+}-free medium or to hypoxia and reoxygenation, which have similar disruptive effects on the cytoskeleton, also caused increases in D_L. Disruption of actin microfilament structure also increased the mobile fraction of Na,K-ATPase. Using a confocal laser microscopic technique only 14.9% of total Na,K-ATPase was observed to reside in the apical membrane domain of normal cells. Microfilament depolymerization caused this fraction to increase to 47.7%. Thus, the translocation of Na,K-ATPase from the basolateral to the apical domain induced by cytoskeletal protein dysfunction was enabled by an increased rate of lateral diffusion of Na,K-ATPase. The behavior of a variety of membrane lipids following actin depolymerization was more heterogeneous. Some lipids showed a similar increase in D_L, whereas others showed very little dependence upon the cytoskeleton for lateral restraint.

Key words: Membrane fluidity — Actin — Cytochalasin D — Epithelial cell — Lipids — Lateral diffusion

Introduction

The cytoskeleton has a number of important functions including defining cell shape, stabilizing the cell membrane, participating in cell movement and cell-substratum attachment, and determining the membrane localization of some membrane proteins. One of these membrane proteins, Na,K-ATPase, is of extraordinary importance to renal tubule epithelial cells because transcellular transport of solutes and of glucose, amino acids, and organic ions is dependent upon the basolateral location and function of this transporter [10]. The predominant basolateral location of Na,K-ATPase is maintained by the cytoskeleton [10]. The α-subunit of the transporter binds to ankyrin which, in turn, is linked to actin [27, 28].

Molitoris has demonstrated in a series of elegant studies that ischemia causes early, profound, duration-dependent and reversible changes in cytoskeletal function [13, 24]. Loss of renal epithelial cell polarity after ischemia, defined as the translocation of Na,K-ATPase from a basolateral to an apical membrane site, has been linked to cytoskeletal dysfunction. The failure of cytoskeletal integrity is mediated, at least in part, by ATP depletion [25].

In a previous study we demonstrated an important influence of the actin cytoskeleton upon the rate of lateral translation of a phosphatidylethanolamine analogue [30]. Either cytochalasin D, a Ca^{2+}-free medium, or hypoxia and reoxygenation produced large increases in the lateral diffusion coefficient (D_L) of the lipid probe. Because cytoskeletal regulation of the distribution of a transmembrane protein such as Na,K-ATPase is well recognized, we sought to determine whether the cytoskeleton also limits the mobility rate of this membrane protein. We also sought to confirm the effect of cytoskeletal disruption on the distribution of Na,K-ATPase, and to quantitate these effects using confocal microscopy. Finally, we endeavored to better define the role of the cytoskeleton in regulating the movement of membrane lipids, as well as proteins, by observing the effects of cytoskeletal disruption on the lateral diffusion of a number of structurally dissimilar lipid fluoroprobes. The rate of lateral mobility of membrane lipids and proteins was measured in individual, intact, attached cells by quantitation of fluorescence redistribution after photobleaching (FRAP) [14, 15].

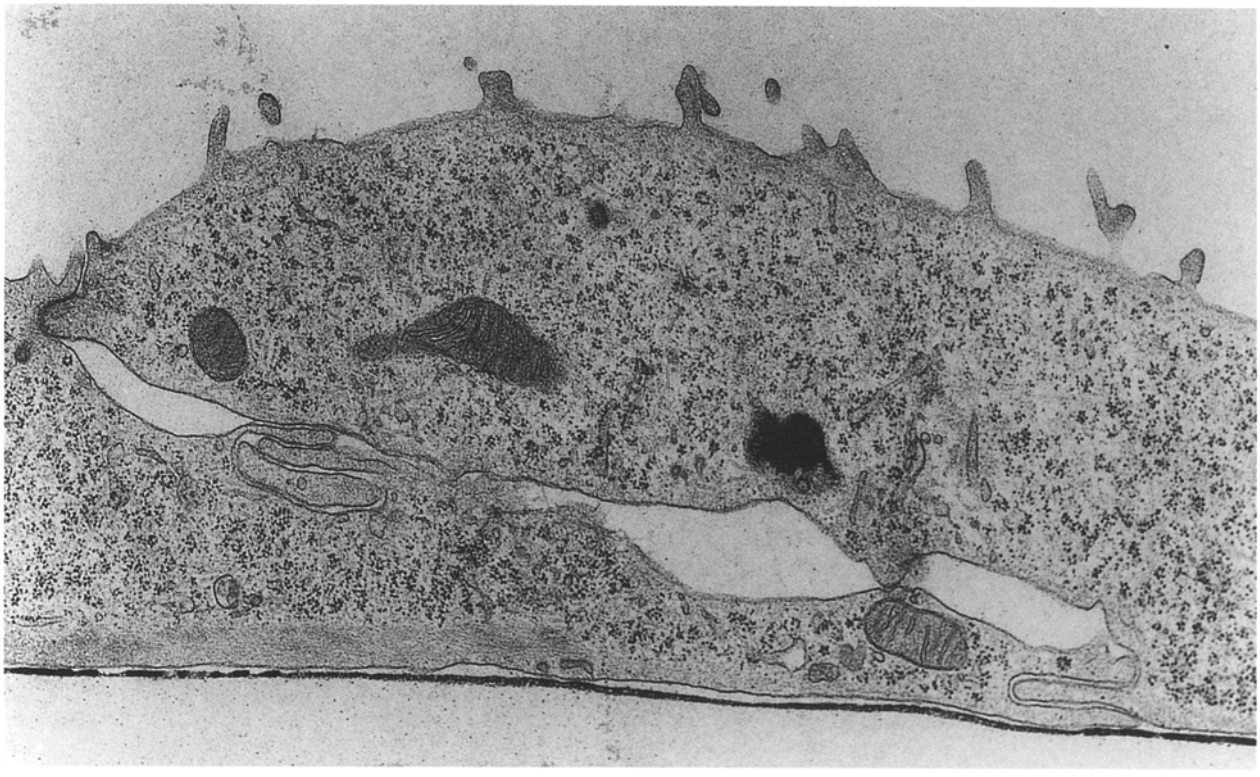

Fig. 1. Epithelial cells (two) of proximal tubule origin grown on collagen-coated plastic dishes for 72 hr. As demonstrated here, these cells form tight junctions and develop interdigitations of lateral membranes when cells make contact. Also note the apical microvilli and basal collection of intermediate filaments, other signs of cell polarity, as well as a well-developed rough endoplasmic reticulum and numerous polyribosomes. The dark material beneath the cell is the collagen gel (uranyl acetate-lead citrate 25,110×).

Materials and Methods

Cell Culture

Rat renal proximal tubule segments were isolated by collagenase digestion of the renal cortex followed by Percoll density gradient centrifugation [31]. This procedure yielded a preparation primarily consisting of proximal tubule fragments (>95%) with approximately 90% viability by vital dye exclusion. Culture medium was RPMI 1640 containing amino acids and vitamins, and (in mM) 11 glucose, 1 Ca $(NO_3)_2$, 5.4 KCl, 0.4 $MgSO_4$, 103 NaCl, 5.6 Na_2HPO_4, 23.8 $NaHCO_3$, and 10 Hepes to which 10% fetal calf serum, 100 μU/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml epidermal growth factor, 5 μg/ml transferrin, 5 μg/ml insulin and 10^{-8} M dexamethasone (final concentrations) were added. Tubule fragments were suspended in culture medium and plated onto collagen gel-coated (Type I, Sigma Chemical, St. Louis, MO) plastic 60 × 15 mm plastic tissue culture plates. Primary cultures were used for all studies.

The proximal origin of the cultured cells was supported by studies which showed expression of proximal tubule brush border enzymes and formation of domes, evidence of vectorial transport. Transmission electron microscopy was performed on cells grown in collagen-coated plastic plates for three days. Cells were fixed in 2.5% phosphate-buffered glutaraldehyde for 1 hr, and post-fixed in 1.5% buffered osmium tetroxide solution for 30 min. They were dehydrated in graded alcohol, transferred to acetone, and embedded in resin (Poly/Bed 812, Polysciences, Warrington, PA). Ultrathin sections were mounted on a copper grid and stained with uranyl acetate and lead citrate, and examined with a Philips 201 electron microscope. These studies revealed apical microvilli and abundant mitochondria, particularly in the basolateral aspects. These cells also formed tight junctions and had intermediate filaments along their basal membranes, other characteristics of polarized epithelium (Fig. 1). Confluent monolayers assayed in situ for the brush border enzyme alkaline phosphatase using a cytochemical assay revealed virtually 100% of cells staining for alkaline phosphatase [31]. Cells were also positive for gamma-glutamyltranspeptidase activity, another proximal tubule brush border marker, by a spectrophotometric assay [31]. Cells were studied in a subconfluent stage, usually three days after initial plating. Forty-eight hours before study, the culture medium was switched to a glucose-free formulation of the usual culture medium to enhance cell susceptibility to hypoxia/reoxygenation injury [31]. At the time of cell labeling with the fluorescent lipid probe, the medium was changed to glucose-free medium without added growth factors or serum.

Fluorescent Cell Labeling

The fluorescent ouabain analogue, 9-anthroylouabain (9-AO) was used to identify Na,K-ATPase [8]; 9-AO was dissolved first in a minimal volume of absolute ethanol and then in RPMI 1640 to yield a 100× stock solution and then added to cells to yield a final concentration of 10^{-7} M. This concentration of 9-AO was sufficient to exceed the saturable binding of the probe by the basal membrane which occurred at $\leq 10^{-8}$ M (determined by confocal microscopic determination of fluo-

rescence in the basal membrane when cells were exposed to 9-AO for 15 min in concentrations between 10^{-10} and 10^{-4} M. After 15 min, the cells were washed three times and then studied for lateral mobility of the Na,K-ATPase. At this concentration there was no toxicity (LDH release) due to inhibition of Na,K-ATPase. When additional experimental manipulations were made (e.g., addition of cytochalasin D to disrupt actin microfilaments), cells were exposed to the 9-AO during the final 15 min of the experimental period.

For comparison purposes, the lateral diffusion rates of several membrane lipids were also determined. NBD-phosphatidylethanolamine (1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazol)-aminocaproyl phosphatidylethanolamine; NBD-PE), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), 6-hexadecanoyl-2-(((2-(trimethylammonium)ethyl)amino)naphthalene (PATMAN), 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine ($DiIC_{12}$), and 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine ($DiIC_{16}$). The lipid probes were incorporated in plasma membranes by exchange in a modification of reported methods [35]. NBD-PE was initially dissolved in chloroform/ethanol (1.2:1 v/v) then added to incubation medium to yield a final concentration of 500 μM. The solution was sonicated for 30 min to disperse the lipid in the aqueous medium, and then cells were incubated with the NBD-PE at 37°C for 60 min. All other lipid probes were first dissolved in absolute ethanol to make up a 100× solution that was than added directly to the edge of the medium overlying the cells and gently agitated. Labeling was performed at 37°C for 15 min using the following final concentration: TMA-DPH 1 mM, PATMAN 5 μM, $DiIC_{12}$ and $DiIC_{16}$ 10 μM. Control cells were exposed to the vehicle (absolute ethanol diluted in the incubation medium) under the same conditions. The various lipid probes were chosen because they distribute to different portions of the lipid bilayer. NBD-PE localizes to both the inner and outer leaflet of the bilayer, as do the lipophilic carbocyanines, whereas TMA-DPH and PATMAN would distribute to the outer portions of the exoplasmic leaflet. All fluorescent cell probes were obtained from Molecular Probes (Eugene, OR).

Fluorescence Redistribution after Photobleaching

The lateral diffusion rate of membrane proteins or lipids was determined by FRAP using an ACAS 570 laser cytometer (Meridian Instruments, Okemos, MI). The ACAS 570 is a fluorescence imaging instrument based on an Olympus IMT-2 inverted phase contrast epifluorescence microscope [33]. A computer-controlled mechanical stage is used to move the specimen in the x and y directions in 0.1 to 18 μm steps. The ACAS 570 has a UV-enhanced 5 W argon ion laser used for excitation in a stationary beam configuration controlled by an acoustically driven beam splitter. The argon ion laser was tuned to 351–363 nm (488 nm for the lipid probes except TMA-DPH) and emissions above 390 nm (550 nm for lipid probes except TMA-DPH) were quantitated. The ACAS 570 system software was used to perform the FRAP studies and to calculate lateral diffusion rates [14, 15, 33]. A limitation of this system is that the image analysis portion of the software program must be able to identify one lateral border of the cell under study so as to distinguish between cellular and extracellular (background) fluorescence. In practice, cells in the midst of a cluster of confluent cells are less suitable for FRAP study than are those at the periphery. The two cells shown in Fig. 1 were at the periphery of a small cluster of cells.

To perform the actual studies, cells were transferred to the temperature-controlled (25°C) stage of the laser cytometer. The optics were adjusted to limit uniform photobleaching during image acquisition to less than 5% of basal fluorescence. Cells were scanned using a 0.2 μm step size and a 100× objective with a numerical aperture of 1.30. An individual cell was scanned to obtain a fluorescent image and the location of the photobleach spot was set using a "mouse and pointer" system. After obtaining three, rapid linear baseline scans of the cell, a 1.3 μm radius spot was "bleached" using a high intensity setting of the laser. Recovery of membrane fluorescence in the bleached area was quantitated by scanning across the cell at 0.6 sec intervals for 72 sec. After the images were corrected for the low levels of background (extracellular) fluorescence, the data were plotted for display by nonlinear least squares fit of the data and the lateral diffusion coefficient (D_L) was calculated from the rate of decay of μ (where μ = 1 minus the minimal value of fluorescence after photobleaching) [14]. This analysis is based on the work of Koppel who described the relationship between fluorescence F at the scanning point Δx from the center of the bleach spot at time t by the following equations:

$$F(\Delta x,t) = F_0\{1 - \beta\alpha_2(t)\exp[-(\Delta x)^2/w^2(t)] - (1-\beta)\alpha_0\exp[-(\Delta x)^2/w_0^2]\} \quad (1)$$

$$\alpha_2 = \alpha_0/(1 + t/\tau_D) \quad (2)$$

$$\tau_D = w_0^2/4D \quad (3)$$

where β represents the size of the mobile fraction, α_0 is a constant that characterizes the extent of bleaching, w_0 is the $1/e$ radius of the laser beam size, τ_D is the time for diffusion, and D is the diffusion coefficient, which can be solved for [14].

To isolate the apical or basal membrane for FRAP studies, confocal imaging was used. First, the microscope was focused above (apical) or below (basal) the nuclear bulge. Then, fluorescent imaging was performed with a 100 μm pinhole aperture placed in front of the photomultiplier tube to decrease the thickness of the cell section being imaged. Cells selected for FRAP measurements after treatment with cytochalasin D, Ca^{2+} removal, or hypoxia and reoxygenation were those that appeared most "normal" by light microscopy. Cells with partial detachment from the collagen substrate, extensive nuclear condensation, extensive cytoplasmic blebs, or any major alteration in cell shape were not evaluated. Unfortunately, it was not technically feasible to perform FRAP studies on hypoxic cells not yet reoxygenated; cells were subjected to both hypoxia and reoxygenation. With this methodology it is not possible to perform FRAP studies in the z-axis so that any Na,K-ATPase in the lateral membrane, an important location of Na,K-ATPase under normal conditions, is inaccessible.

Distribution of Na,K-ATPase between the membrane domains was similarly quantitated. After labeling cells with 9-AO, fluorescent images of both the apical and basal membrane were obtained using confocal microscopy with the pinhole aperture set at 75 μm. The z width at half maximum (an index of optical thickness) for the objective, excitation/emission wavelength, and pinhole setting was 0.53 μm. The average fluorescence intensity for each image was determined from emissions detected by the photomultiplier tube, digitized and processed by the 16-bit microcomputer of the ACAS 570 system [33]. The fraction of fluorescence detected in the apical membrane (proportional to the density of Na,K-ATPase) relative to total fluorescence (apical + basal) was calculated. These studies also served the secondary purpose of demonstrating that 9-AO gained access to the basal surface of the cell under all experimental conditions, including baseline.

Effects of Disruption of the Cytoskeleton

To examine how disruption of actin microfilaments would affect the *rate* of lateral translation as well as the membrane distribution of Na,K-ATPase, cells were exposed to cytochalasin D to depolymerize actin filaments. Cells were treated with cytochalasin D (10 μM; Sigma) for 60 min during the cell labeling period [5, 13]. Because the cytochalasin D was first dissolved in dimethyl sulfoxide, an additional control

group was studied to be sure that a final concentration of dimethyl sulfoxide of 1% by volume had no independent effect on Na,K-ATPase diffusion. Alternatively, to disrupt cell junctions and to induce microfilament and microtubule retraction, the incubation medium was changed to a nominally Ca^{2+}-free formulation after cell labeling was completed [19, 21]. The "Ca^{2+}-free" medium was not treated with EGTA or other calcium chelators and had an ionized Ca^{2+}-free content of 2.11×10^{-6} M calcium when assayed by flameless atomic absorption spectroscopy (Twin Cities Testing, Minneapolis, MN). Neither treatment caused irreversible cell injury, as evidenced by a lack of increased LDH release during the experiment compared with control cells. Under phase microscopy cytochalasin-D-treated cells showed rounding up or folding up of their edges and loss of the normal polygonal shape as well as an increase in the intercellular spaces (Fig. 2). A small number of cells instead had a "filmy" or indistinct cell outline due to cytoplasmic spreading. There was no obvious cell fragmentation. Cells exposed to Ca^{2+}-free medium were also rounded up and had markedly increased intercellular spaces and changes. They did not have membrane blebbing or cell fragmentation.

The third mechanism of interfering with cytoskeletal function involved subjecting the cells to hypoxia and reoxygenation [30]. To produce hypoxia/reoxygenation injury, cell plates were placed in an airtight glass chamber under a continuous flow of humidified gas and maintained at 37°C: hypoxia (95% N_2, 5% CO_2) for 60 min followed by reoxygenation (95% O_2, 5% CO_2) for 30 min. This protocol was determined previously to produce hypoxia followed by reoxygenation without the development of hyperoxia during the reoxygenation phase [31]. Control cells were studied after incubation under normoxic conditions (95% air, 5% CO_2) for 90 min. Cell viability was determined by measuring the release of lactate dehydrogenase (LDH) into the incubation media which correlates with critical cellular injury and loss of other cytoplasmic constituents in cells which have lost cellular integrity. Incubation medium was promptly aspirated with a Pasteur pipette and LDH was determined as the increase in NADH (absorbance at 339 nm at 30°C, extinction coefficient of 6.30 mM^{-1} cm^{-1}) in the presence of 6.5 mM β-NAD and 52 mM L-lactate in 100 mM Tris, pH 9.3. Cellular LDH was determined after scraping the cells from the tissue culture plate, disrupting the cell membranes by mechanical homogenization, and then measuring LDH in the resulting homogenate. LDH release was expressed as the percent of total cellular LDH (supernate plus cell fraction) recovered in the incubation medium.

Statistical Analysis

All data are shown as mean ± SE. Statistical comparison was performed by unpaired *t*-test when two groups were compared and when multiple groups were compared critical values for *t* were determined by the Bonferroni method.

Results

The lateral diffusion coefficient of Na,K-ATPase in basal membranes was $3.31 \pm 0.7 \times 10^{-10}$ cm^2/sec. In normal cells there was not sufficient Na,K-ATPase in apical membranes to perform FRAP studies of that membrane domain. Photobleaching reduced fluorescence by approximately 50%. The fraction of labeled Na,K-ATPase that was mobile was 47.0 ± 4.1%. Disruption of the actin cytoskeleton, a common feature of cytochalasin D treatment, removal of extracellular Ca^{2+}, or exposure to hypoxia and reoxygenation, had profound effects on the lateral diffusion rate of Na,K-ATPase. In basal membranes, cytochalasin D produced a sevenfold increase in D_L of Na,K-ATPase (Table 1). Ca^{2+}-free medium caused a similar change in the lateral diffusion rate of Na,K-ATPase, producing an approximately fourfold increase in D_L (Table 1).

Hypoxia and reoxygenation was also found to increase the lateral mobility of Na,K-ATPase. Hypoxia and reoxygenation irreversibly injured more than one-third of the cells; LDH release was 23.6 ± 2.9% for control cells and 56.3 ± 5.6% after hypoxia/reoxygenation ($P < 0.001$). The most normal appearing cells (by phase microscopy), rather than those with extensive membrane blebs or focal disruption of the plasma membrane, were selected for determination of D_L. D_L increased almost threefold following reoxygenation injury (Table 1).

Actin depolymerization also had the predicted effect on the distribution of Na,K-ATPase [26]. In normal cells 14.9 ± 1.7% ($n = 24$) of labeled Na,K-ATPase was found in the apical membrane (relative to apical plus basal). Because the lateral domains were excluded from this analysis, this determination overestimates the fraction of total membrane Na,K-ATPase found in the apical domain. After cytochalasin D treatment, the apical portion increased to 47.7 ± 0.9% ($n = 13$; $P < 0.0001$). Cytochalasin D caused no change in the very low level of cell autofluorescence (determined in the absence of 9-AO) and, therefore, did not interfere with quantitation of Na,K-ATPase localization. Associated with this translocation of Na,K-ATPase to the apical domain was an increase in the mobile fraction of ouabain-labeled Na,K-ATPase to 62.6 ± 7.7% ($P < 0.05$ *vs.* control) as determined during the FRAP studies.

The effect of actin depolymerization on the lateral diffusion rates of several different lipid probes was also tested. The results of these experiments are summarized in Table 2. Compared with Na,K-ATPase, lipid D_L was one order of magnitude greater, except for NBD-PE which has a D_L similar to that of Na,K-ATPase. For most of these lipid probes, D_L was greater in the basolateral than apical membrane, as expected [7, 30]. Cytochalasin D caused increases in apical D_L of TMA-DPH and NBD-PE and no change for PATMAN. Quite unexpectedly, actin microfilament depolymerization had the opposite effects on D_L of the two carbocyanine probes, $DiIC_{12}$ and $DiIC_{16}$. These effects were compared to those of temperature change on D_L of $DiIC_{16}$. There was no significant changes in D_L between 22°C ($D_L = 43.7 \pm 9.7 \times 10^{-10}$ cm^2/sec) and 37°C ($D_L = 45.3 \pm 12.2 \times 10^{-10}$ cm^2/sec). The effects of temperature on D_L of the other lipid probes was not evaluated. Cytochalasin D caused small but significant decreases in the lateral mobility of these two probes. Actin depolymerization also had no effect on the mobile fraction of the lipids, except in the case of $DiIC_{12}$ where the mobile

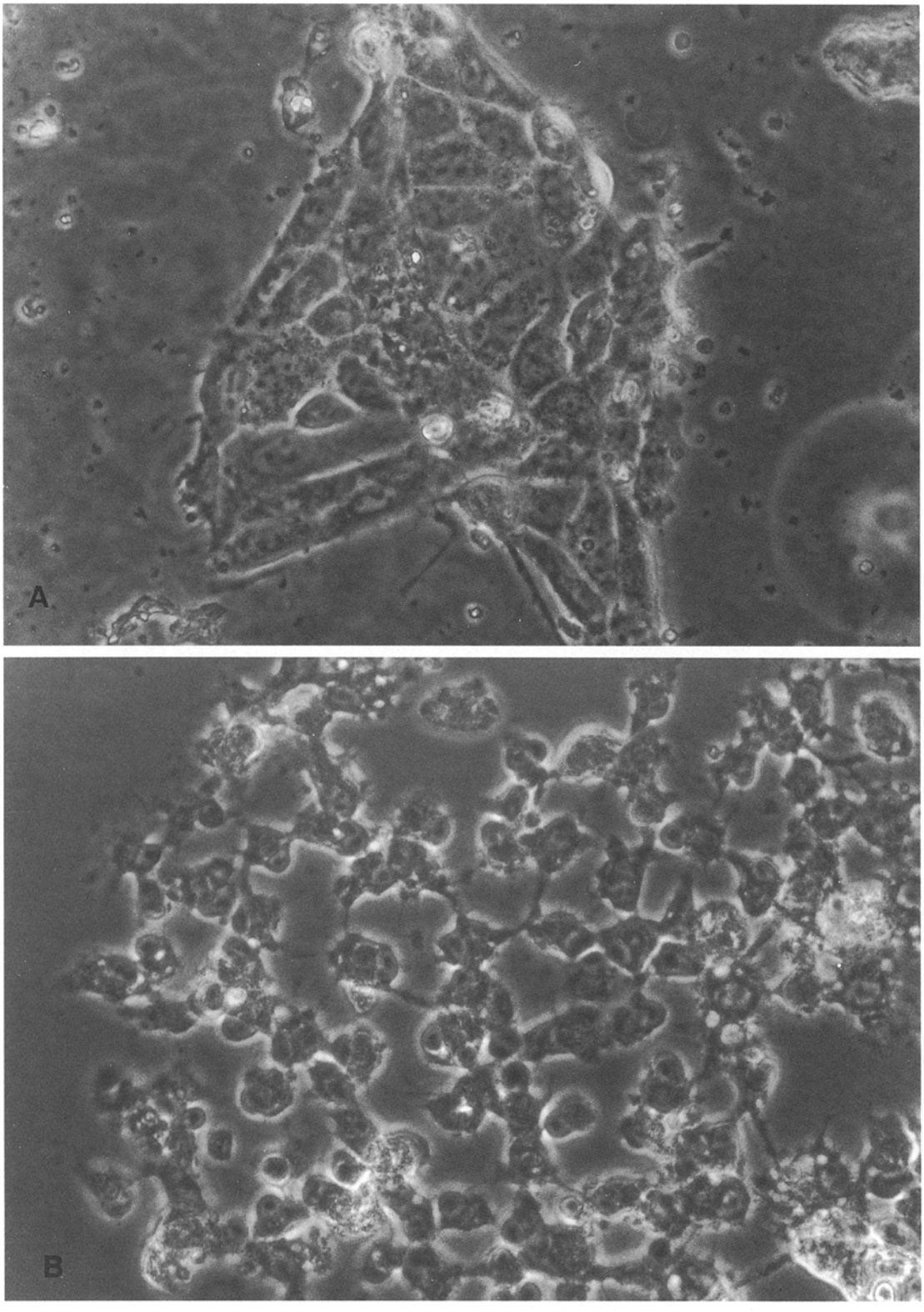

Fig. 2. Rat proximal tubule epithelial cells in primary culture. (*A*) A cluster of cells under control conditions. Subconfluent cells were used for all studies (*see text* for details). (*B*) Cells exposed to cytochalasin D (10 μM × 60 min) demonstrate rounding up, loss of cell-cell contacts, and increased intercellular spaces (phase contrast 100×).

Table 1. Effect of cytoskeletal perturbations on the lateral diffusion of Na,K-ATPase in the basal membrane

Condition	(*n*)	D_L ($\times 10^{-10}$ cm^2/sec)	Recovery (%)
Control	10	3.31 ± 0.7	47 ± 4.1
Cytochalasin D	10	23.9 ± 7.8[a]	62.6 ± 5.7[d]
Ca^{2+}-free medium	25	11.9 ± 3.2[b]	59.6 ± 7.7
Hypoxia/reoxygenation	19	8.1 ± 1.2[c]	55.7 ± 5.4

9-AO-labeled cells were studied by the FRAP technique under control conditions, exposure to cytochalasin D (10 μM for 60 min), exposure to a nominally Ca^{2+}-free medium, or after exposure to 60 min of hypoxia and 30 min of reoxygenation. D_L, lateral diffusion coefficient. *n*, number of cells studied. The cells were obtained from a minimum of three separate batches of cells studied on different days. [a]$P = 0.009$ *vs.* control; [b]$P = 0.09$ *vs.* control; [c]$P < 0.01$ *vs.* control.

Table 2. Lateral mobility of several lipid probes and the effect of cytochalasin D

Lipid probe	Apical D_L	Basolateral D_L	Apical D_L after cytochalasin D
TMA-DPH	26.4 ± 3.1	29.2 ± 4.9	53.9 ± 14.0[b]
PATMAN	10.3 ± 2.4	22.7 ± 5.4[b]	11.7 ± 1.2
$DiIC_{12}$	37.9 ± 6.7	51.0 ± 9.9[b]	21.2 ± 2.6[b]
$DiIC_{16}$	33.2 ± 4.6	46.7 ± 4.5[b]	11.8 ± 4.6[b]
NBD-PE[a]	3.2 ± 0.8	6.6 ± 1.2[b]	23.5 ± 6.5[b]

Cells labeled with the various lipid fluoroprobes (*see text* for details) were studied by the FRAP technique. D_L, lateral diffusion coefficient ($\times 10^{-10}$ cm^2/sec). Cells were obtained from a minimum of three separate batches studied on different days and at least 20 cells were studied for each condition. [a]Data reported in reference [23]. [b]$P < 0.05$ (or less) *vs.* control apical D_L.

fraction fell from 55.4 ± 2.6 to 45.3 ± 2.3% ($P < 0.02$). By comparison, the mobile fraction of the structurally similar $DiIC_{16}$ was 60% before and after cytochalasin D.

Discussion

The cytoskeleton has profound effects on many membrane proteins and lipids, both in terms of their distribution and of their lateral mobility. Most lipids in mammalian cells have translational diffusion rates of 10^{-9} to 10^{-8} cm^2/sec [7, 11, 15]. Lateral diffusion rates for membrane proteins are usually one to two orders of magnitude slower. The lesser translational motion rates for proteins are believed to be secondary to restricted movement by the cytoskeleton. For example, in normal erythrocytes lipid lateral diffusion rates were 300-fold greater than that for a membrane protein [15]. Spectrin-deficient erythrocytes had protein lateral diffusion rates one-sixth that for lipid or 50-fold greater than in normal erythrocytes with an intact cytoskeletal matrix. Similarly, spectrin dissociation (induced by lowering the ionic strength) increased the mobile fraction and rate of lateral diffusion of erythrocyte band 3 protein [9]. Thus, protein diffusion may be sterically hindered by the submembrane matrix.

Na,K-ATPase is restricted to a basolateral location through binding to actin and associated actin-binding proteins [10]. When cytoskeletal-membrane protein binding is disrupted by ischemia or ATP depletion, Na,K-ATPase is freer to translocate to the apical membrane [23, 25]. In the current study, we have extended these observations by demonstrating that depolymerization of actin microfilaments can induce the same translocation of Na,K-ATPase. We cannot rule out the possibility that cytochalasin D resulted in "unmasking" of Na,K-ATPase already present on the apical membrane, but think that possibility is unlikely. Total fluorescence (apical plus basal) did not change after cytochalasin D; if apical "unmasking" had occurred, basal "masking" would have also needed to occur in parallel. Moreover, binding of Na,K-ATPase to actin-associated proteins is a dynamic rather than static process. Using a fluorescent oubain analogue to monitor the location and movement of Na,K-ATPase, FRAP studies revealed that approximately half of the ouabain-binding Na,K-ATPase was freely mobile within the time frame of the experiment (72 sec). This observation is similar to that of Jesaitis and Yguerabide [11] whose measurement of D_L of Na,K-ATPase in a renal epithelial cell line was nearly identical to the value determined here in primary cultured cells. This finding suggests that static binding of Na,K-ATPase to the actin cytoskeletal complex is not sufficient to restrict almost all Na,K-ATPase in the plasma membrane to the basolateral domain. When cells were treated with cytochalasin D to induce actin filament depolymerization, the fraction of mobile Na,K-ATPases increased by about 33%. At the same time, the rate at which the mobile Na,K-ATPases diffused laterally increased greatly. Depolymerization of actin by cytochalasin D caused a sevenfold increase in the lateral diffusion coefficient of Na,K-ATPase. These findings also fit nicely with the observations of Madreperla et al. [18] that as retinal photoreceptor cells develop, establishment of cellular Na,K-ATPase polarity is inversely correlated with the fraction of mobile ATPase molecules and positively correlated with the association of ATPase with spectrin in the cytoskeleton. We believe these two factors contributed to the striking translocation of Na,K-ATPase from the basolateral to the apical domain when the actin cytoskeleton was not intact. Therefore, an enhanced rate of movement as well as reduced binding of Na,K-ATPase enabled its redistribution when cytoskeletal restriction of Na,K-ATPase was impaired.

In an effort to better understand the dynamic nature of cytoskeletal-membrane interactions, we evaluated the effect of actin cytoskeleton disruption on the lateral mobility of several membrane lipid probes. We had previ-

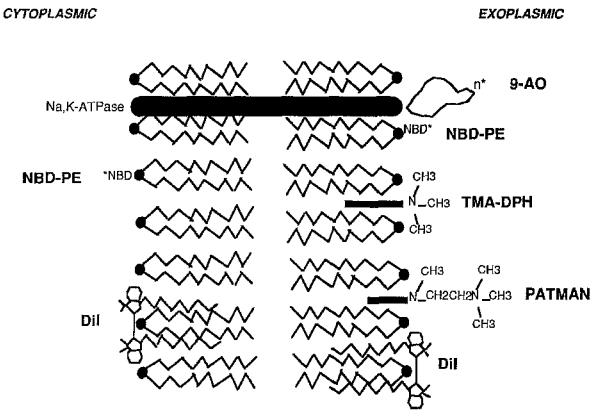

Fig. 3. Probable membrane distribution of fluoroprobes used in these studies. 9-AO binds to the transmembrane protein Na,K-ATPase. The phospholipid probe NBD-PE distributes to both the exoplasmic and cytoplasmic leaflet of the lipid bilayer of the plasma membrane. The polar probes TMA-DPH and PATMAN are probably confined to the polar region of the outer leaflet, whereas the carbocyanines distribute to nonpolar regions of both leaflets.

ously observed that actin cytoskeletal disruption or dysfunction produced a marked increase in the lateral diffusion coefficient of the phosphatidylethanolamine analogue NBD-PE [30]. We extended these studies by using probes with differing distribution within the lipid bilayer (cytoplasmic *vs.* exoplasmic leaflet) and variable penetration within the bilayer dependent upon the polarity of the fluoroprobe [1, 4, 16, 32, 39] (Fig. 3).

It is well recognized that D_L differs depending on the lipid studied [7, 36]. This is readily apparent by examining Table 2. Most of the probes examined had D_L in the 10^{-9} cm^2/sec range, similar to values reported in other studies [2, 7, 34]. The striking exception was NBD-PE which had a lateral diffusion coefficient one order of magnitude slower. NBD-PE also has striking limitation of lateral mobility due to cytoskeletal influences. As we previously reported, cytoskeletal disruption with cytochalasin D increased D_L of NBD-PE nearly 10-fold [28], to a rate much closer to that characteristic of the other lipid probes. A phosphatidylethanolamine (PE) derivative was initially chosen for study because PE is almost equally abundant in apical and basolateral membranes, whereas PC and PI, for example, are preferentially distributed in the basolateral domain [3].

Structure-function analysis does not readily explain the different behavior of the various lipid probes although it suggests one explanation for the slower diffusion of transmembrane proteins compared with most membrane lipids. As Fig. 3 diagrams, membrane proteins like Na,K-ATPase span the entire lipid bilayer. The bulky protein could be restricted in its lateral diffusion because of varying lipid composition and physical properties of the two leaflets of the bilayer. This restriction would be in force even when cytoskeletal tethers were broken, as occurs after cytochalasin D treatment. A similar phenomenon holds for membrane spanning lipids. Vaz et al. [39] found that a nonmammalian membrane-spanning phosphatidylethanolamine had lateral mobility two-thirds that of phosphatidylethanolamine confined to a single leaflet (the usual situation). It is more difficult to explain exactly why the lipid probes differ so widely in their behavior, except that size and charge of hydrophilic regions as well as length and saturation of fatty acyl chains will affect the lipid's ability to move amongst its neighbors [2, 12, 20, 34].

The effects of cytoskeletal dysfunction on protein lateral diffusion were not surprising based on the known binding of Na,K-ATPase to actin-associated proteins. The effects of cytoskeletal dysfunction on lipid lateral diffusion were neither easily predictable nor uniform. As already noted, we previously observed an increase in the lateral diffusion rate of the phospatidylethanolamine analogue NBD-PE [30]. If this were a uniform response it would have held for all of the lipid probes tested in these experiments. As can be easily seen in Table 2, cytochalasin D also increased D_L for TMA-DPH but decreased it for the two carbocyanines, $DiIC_{12}$ and $DiIC_{16}$. There was no change in D_L for PATMAN, which is perhaps surprising since it might be predicted to behave similar to TMA-DPH. The unknown factor here is whether (and if so, to what extent) the various lipid probes bind to cytoskeletal proteins [6, 29]. Although actin probably does not insert directly into bilayers, several actin binding proteins do have direct interactions with lipids [29]. The ability of cytochalasin D to decrease D_L for $DiIC_{12}$ and $DiIC_{16}$ is difficult to understand whether or not there is any direct linkage between the probe and the actin cytoskeleton. This differential effect again points to the importance of lipid heterogeneity and of lipid microdomains in contributing to the net characteristics of the plasma membrane.

In the current studies we have also confirmed that basolateral membrane lipid D_L was approximately twice as great as in apical membranes, an observation previously made by Dragsten et al. using a similar FRAP technique and by this laboratory using NBD-PE [7, 30]. Measures of other parameters of membrane fluidity in renal cell apical or basolateral membrane vesicles have also revealed greater basolateral fluidity [17, 22].

The potential importance of these findings is clarified by examining the hypothesis that tubular cell dysfunction after injury (in terms of solute reabsorption) is a consequence of the cytoskeleton-dependent loss of epithelial cell polarity [22, 25]. The loss of Na,K-ATPase polarization due to cytoskeleton dysfunction allows the protein to dissociate from cytoskeleton-protein linkages and to then diffuse into the apical domain [25]. Increased lateral diffusive movement of Na,K-ATPase and of lipids, as well, would speed this redistribution of membrane components. The increase in the mobile frac-

tion of membrane Na,K-ATPases we observed would also contribute to the total pool of translocatable Na,K-ATPase. Ischemia-induced histologic changes, such as bleb formation, have also been attributed to impaired cytoskeleton function [37]. Enhanced diffusive movement of plasma membrane components could be an integral aspect of these pathologic changes also. Therefore, these findings give additional support for the role of the actin cytoskeleton to importantly regulate membrane distribution of Na,K-ATPase (and some membrane lipids) in dynamic terms.

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