

The Lateral Mobility of the (Na⁺,K⁺)-dependent ATPase in Madin-Darby Canine Kidney Cells

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Abstract. Fluorescence microphotolysis (recovery after photobleaching) was used to determine the lateral mobility of the (Na⁺,K⁺)ATPase and a fluorescent lipid analogue in the plasma membrane of Madin-Darby canine kidney (MDCK) cells at different stages of development. Fluorescein-conjugated Fab' fragments prepared from rabbit anti-dog (Na⁺,K⁺)ATPase antibodies (IgG) and 5-(N-hexadecanoyl)amino-fluorescein (HEDAF) were used to label the plasma membrane of confluent and subconfluent cultures of MDCK cells. Fractional fluorescence recovery was 50% and 80–90% for the protein and lipid probes, respectively, and was independent of developmental stage. The estimated diffusion constants of the mobile fraction were $\sim 5 \times 10^{-10}$ cm²/s for the

(Na⁺,K⁺)ATPase and $\sim 2 \times 10^{-9}$ cm²/s for HEDAF. Only HEDAF diffusion showed dependency on developmental stage in that D for confluent cells was approximately twice that for subconfluent cells. These results indicate that (Na⁺,K⁺)ATPase is 50% immobilized in all developmental stages, whereas lipids in confluent MDCK cells are more mobile than in subconfluent cells. They suggest, furthermore, that the degree of immobilization of the (Na⁺,K⁺)ATPase is insufficient to explain its polar distribution, and they support restricted mobility of the ATPase through the tight junctions as the likely mechanism for preventing the diffusion of this protein into the apical domain of the plasma membrane in confluent cell cultures.

THE (Na⁺,K⁺)-dependent ATPase (EC3.6.1.3) is an integral plasma membrane protein that catalyzes activated co-transport of Na⁺ out and K⁺ into cells coupled with the hydrolysis of ATP (39). The enzyme consists of an α , β dimer of 110 and 50 kD, respectively (20). This structural unit, referred to as the α , β protomer, is believed to also be the smallest functional unit of the enzyme capable of ion transport (10).

Structural studies of this protein suggest that the α , β protomer is 115 Å in its longest direction, spanning the membrane and at most ~ 50 – 60 Å in diameter in a plane parallel to the membrane (44). The extracellular face of the protomer protrudes ~ 20 Å from the bilayer. The intracellular face protrudes ~ 50 Å from the bilayer and contains ATP binding sites (7, 25) and functional sulfhydryls significantly removed from the bilayer by ~ 30 – 40 Å (16). These structural studies and others (18, 26) suggest that the α subunit has a sufficiently large cytoplasmic domain to permit it to interact directly with cytosolic proteins. Such interaction could possibly be important in maintaining its distribution in the plasma membrane (1).

Lateral asymmetric distribution of the (Na⁺,K⁺)ATPase has been observed in the plasma membranes of cells in a number of tissues, including those of epithelial, neuronal, and photo-receptor origin (see reference 1). It is believed that such asymmetric distributions are vital for the vectorial ion trans-

port or ionic currents in these cells or for controlling the types of proteins in the membrane with which the enzyme can interact. The mechanism for the establishment and maintenance of such cell polarity is still unknown.

The Madin-Darby canine kidney (MDCK)¹ cell line (35) has been used to investigate cell polarity (23). U and Evans-Layng (40) observed that the (Na⁺,K⁺)ATPase was confined to the apical surface in subconfluent monolayers and is redistributed to the basolateral surface in confluent layers. Several studies of surface proteins in MDCK cells have suggested that the cell polarity is the result of polarized vesicular transport of membrane proteins (6, 22, 24, 36) by membrane flow processes (27). In addition, the maintenance of the polarized distribution has been postulated to be due to restricted movement of the protein through the zona occludens or tight junctions (8, 40, 41). On the other hand, recent studies on the mobility of wheat germ agglutinin receptors in MDCK cells (11) indicated that these receptors were completely immobile on the basolateral and apical surfaces. This latter result suggested that cell polarity might be maintained via restricted mobility of the membrane proteins themselves. The inference is supported by evidence that indicates some proteins appear to pass through the zona occludens (14, 34), which suggests

¹ Abbreviations used in this paper: FITC, fluorescein isothiocyanate; FRAP, fluorescence recovery after photobleaching; HEDAF, 5-(N-hexadecanoyl)aminofluorescein; MDCK, Madin-Darby canine kidney.

that the zona occludens does not impede the lateral mobility of at least some membrane proteins.

To gain further insight into the mechanism of control of cell polarity, we have measured the lateral mobility of the $(\text{Na}^+, \text{K}^+)\text{ATPase}$, a wheat germ agglutinin binding protein (30), in MDCK cells using specific monovalent Fab' antibody probes. We report the results of the fluorescence microphotolysis (i.e., fluorescence recovery after photobleaching [FRAP]; see references 32 and 43) measurements of the lateral mobility of the $(\text{Na}^+, \text{K}^+)\text{ATPase}$ in the plasma membrane of confluent and subconfluent cultures of these cells. Our results suggest that the lateral motion of the $(\text{Na}^+, \text{K}^+)\text{ATPase}$ is partially, but not totally restricted, demonstrating a population that is 50% mobile with a diffusion constant of $5 \times 10^{-10} \text{ cm}^2/\text{s}$. This result is to be contrasted with previously reported studies using wheat germ agglutinin as a probe of cell surface mobility, which indicated that wheat germ agglutinin receptors were completely immobile. These results have been previously reported in abstract form (17).

Materials and Methods

Cells

The MDCK cell line was maintained exactly as described in Rindler et al. (35) except that before cell passage and dilution 4–6 clean square cover glasses (22 mm) were placed in the petri dishes to provide a substratum for cell growth that could easily be used for labeling, washing, media transfer, and observation.

Antibodies

Anti-dog $(\text{Na}^+, \text{K}^+)\text{ATPase}$ antisera raised against pure $(\text{Na}^+, \text{K}^+)\text{ATPase}$ (22) was the kind gift of Dr. Daniel Louvard. To prepare the IgG fraction, serum was dialyzed against 10 mM KH_2PO_4 , pH 8.0, overnight with one change of buffer (1 liter/5 ml sera). The dialyzed serum was centrifuged to clarity at 10,000 rpm in a Sorvall SS-34 rotor for 10 min. The supernatant was then applied to a DEAE-cellulose (Whatman 52, Whatman Inc., Clifton, NJ) column equilibrated in dialysis buffer. 60% of the total IgG was collected in the flow through as a pure fraction as judged by homogeneous heavy and light chains by reduced SDS PAGE.

(Fab')₂ fragments were prepared by 3% wt/wt pepsin digestion at pH 4.0 for 18 h at 37°C. This step was followed by dialysis against phosphate-buffered saline (PBS), and centrifugation at 10,000 rpm for 10 min at 4°C. SDS PAGE of the digests did not show undigested IgG. Fab' fragments were prepared by reducing (Fab')₂ in 20 mM dithiothreitol for 2 h at room temperature at pH 8 and then alkylating with 20 mg/ml recrystallized iodoacetamide for 30 min at 4°C. Final Fab' sample was then dialyzed against PBS and was homogeneous in analyses by SDS PAGE.

Fluorescein isothiocyanate (FITC, Sigma Chemical Co., St. Louis, MO) was used to conjugate Fab' fragments of anti-ATPase and normal rabbit serum. Conjugation was performed in a dialysis bag in carbonate buffer at pH 9.0 for 18 h after which the free FITC was dialyzed out against PBS. The conjugate was centrifuged to clarity and chromatographed on a DE-52 column to isolate conjugates with a fluorescein/protein ratio of 3–4:1.

Cell Labeling and Immunofluorescence

To fluorescently label cells for FRAP measurements, coverslips were used from MDCK cultures 1–5 d after passage and dilution. After washing in Dulbecco's PBS + 0.5% bovine serum albumin (BSA), the cells were incubated with FITC-conjugated anti- $(\text{Na}^+, \text{K}^+)\text{ATPase}$ Fab' fragments at 100–400 $\mu\text{g}/\text{ml}$ in Dulbecco's PBS \pm 20 mM NaN_3 for 30 min on ice, followed by two washes in Dulbecco's PBS + 0.5% BSA. Antibody labeling was judged specific in that it was not visible if cells were pretreated with unconjugated antibody or if fluorescein-conjugated (fluorescein/protein ratio of 3–4:1) normal rabbit serum Fab' was used in the labeling procedure.

To label cells for immunofluorescence photography, coverslips with growing MDCK cells were rinsed in PBS two times and fixed in 2% paraformaldehyde for 20 min at room temperature followed by 5-min incubation in 0.1 M glycine in PBS and a wash in PBS. This preparation was then exposed to 100–400 $\mu\text{g}/\text{ml}$ anti- $(\text{Na}^+, \text{K}^+)\text{ATPase}$ IgG and washed two times for 5 min with 4 mg/ml BSA in PBS and one time with PBS. The preparation was then incubated for

5 min at 37°C with tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG antibody and washed again as before. The preparation was then mounted in 90% glycerol in PBS by successive 5-min immersions in 10, 30, 60, and 90% glycerol PBS. Photography was performed on a Zeiss photomicroscope (Carl Zeiss, Inc., Thornwood, NY) using 63×1.4 numerical aperture objective and Kodak Tri-X film processed with Accufine developer.

FRAP

FRAP measurements were done at room temperature as described by Woda et al. (42).

Results

The MDCK epithelial cell line forms monolayers of cells in culture that possess permeability and transport properties similar to in vivo transporting epithelia. After trypsinization of seed cultures, the replated cells grow to form islands of cells which in turn grow to confluence forming large sheets of cells with tight junctions (8, 15, 35). The surfaces of cells in these various stages were fluorescently labeled with HEDAF to assess the mobility of the plasma membrane lipids at different growth stages. If the labeling was done in the presence of NaN_3 , then a greater number of lateral mobility measurements could be made before internal labeling was apparent. Cells labeled with HEDAF were intensely fluorescent and appeared as reported previously by Dragsten et al. (11).

FRAP determinations were made on the apical surfaces of both confluent and subconfluent cells labeled with HEDAF. Fig. 1 and Table I show the results of such determinations. Fluorescence recovery was 80–90% of the intensity before photobleaching in both types of cells. The calculated diffusion constants were 6 and $20 \times 10^{-10} \text{ cm}^2/\text{s}$ for subconfluent and confluent cells, respectively. The latter figure agrees within experimental error with the value $(4 \pm 2 \times 10^{-9} \text{ cm}^2/\text{s})$ reported for HEDAF by Dragsten et al. (11) for the apical surfaces of confluent layers of MDCK cells.

When cells, fixed at different stages of growth, were labeled with FITC-anti-dog $(\text{Na}^+, \text{K}^+)\text{ATPase}$ IgG, it was evident that the distribution of the externally accessible antibody sites depended on the maturity of the culture. Immature cultures that demonstrate isolated islands of MDCK cells are shown in Figs. 2 and 3, *a* and *b*. These islands were brightly fluores-

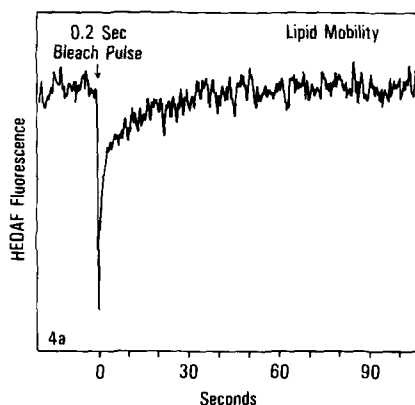


Figure 1. FRAP of HEDAF in the apical surface of a confluent monolayer of cultured MDCK cells. Fluorescence was excited in a 2.5- μm -diam circular spot on the cell surface by a focused He-Cd laser microbeam ($\lambda_{\text{ex}} = 451 \text{ nm}$) and measured through microscope optics by photon counting. From these curves, percent recovery, R , and half time of recovery, $t_{1/2}$, were used to determine the mobile fraction of HEDAF and its lateral diffusion constant, D . The results are summarized in Table I.

Table I. Lateral Mobility of MDCK Cell Plasma Membrane Components

Probe	Cell stage	Azide	Percent recovery	$t_{1/2} \pm SEM$	D	No. trials
			$R \pm SEM$	s	cm^2/s	
HEDAF	Subconfluent	Yes	78 ± 3	8 ± 2	6×10^{-10}	25
	Confluent	Yes	92 ± 2	3 ± 0.5	2×10^{-9}	14
Anti-(Na ⁺ ,K ⁺)ATPase Fab'	Subconfluent	No	51 ± 0.4	26 ± 0.4	2×10^{-10}	59
	Subconfluent	Yes	47 ± 6	12 ± 2	4×10^{-10}	4
	Confluent (basolateral only)	Yes	50 ± 1	10 ± 0.5	5×10^{-10}	16

cent from antibody labeling and showed punctate fluorescence. The labeling of individual cells usually appeared about the same over small cell islands that contained less than 10–15 cells (not shown). As the islands enlarged or coalesced with others, labeling became heterogeneous (Fig. 2, *c* and *e*, and 3*a*) or was observable only at the outer perimeters of the layer (not shown). Fluorescence intensity decreased toward the center of these large clusters.

Fully confluent cells showed virtually no fluorescent labeling unless breaks in the layer were made and the basolateral surfaces were exposed by folding back the edge of a tear (Fig. 3, *c* and *d*). Such regions were the most intensely stained of all the preparations. These results indicate that in the confluent cells, the (Na⁺,K⁺)ATPase is confined to the basolateral surfaces and that cell–cell contact appears to be accompanied by loss of (Na⁺,K⁺)ATPase from the apical surface. The results are consistent with previous observations made by U and Evans-Layng (40) in MDCK cells. Identical distributions have been demonstrated for the 50,000-kD glycoprotein of MDCK cells localized by Herzlinger and Ojakian (15) and therefore suggest that the cells have achieved full polarity.

To evaluate the role of lateral mobility in the establishment and maintenance of asymmetric distribution of (Na⁺,K⁺)ATPase at the cell surface, we made FRAP measurements on cells labeled with FITC-conjugated anti-dog (Na⁺,K⁺)ATPase Fab' fragments. Cells labeled with the probe showed visually bright "ring" fluorescence characteristic of surface labeling. The fluorescence, however, was bleached rapidly by the excitation beam used for photography and was consequently not photographed. Specificity of labeling was therefore established by dark-adapted visual observation using control antibodies. Labeling was inferred to be specific in that it was not visible if cells were pre-exposed to unconjugated anti-dog (Na⁺,K⁺)ATPase Fab' fragments. In addition, labeling was unaffected by exposure to normal rabbit serum Fab' fragments either before or after labeling with specific antibody fragments.

The cells chosen for lateral mobility measurements were ones that clearly demonstrated bright specific fluorescence. These included brightly stained apical surfaces in subconfluent cells. In such subconfluent cell clusters, no attempt was made to label the surfaces attached to the coverslips because the distributions observed were in agreement with those of U and Evans-Layng (40), who reported in their electron microscopic studies that the (Na⁺,K⁺)ATPase was confined only to the apical domain at this stage of culture development. No clear correlation emerged between the mobility properties of the (Na⁺,K⁺)ATPase in cells on the edge of clusters or in the middle of such clusters. Consequently, all the mobility mea-

surements on immature clusters of cells were grouped together and averaged.

In confluent layers, only those cells exposed to labeling by tearing of the monolayer were used for the lateral mobility measurements. These regions were easily discernible because of their bright labeling and characteristic flaplike morphology (see region between arrows in Fig. 3, *c* and *d*). Table I and Fig. 4 show the results of these measurements. Clearly, the (Na⁺,K⁺)ATPase appeared 50% immobilized at the time of measurement. This fraction was also independent of the maturity of the culture and did not require metabolic energy for its maintenance as evidenced by its insensitivity to NaN₃. The calculated diffusion constant of the mobile fraction (5×10^{-10} cm²/s) was, however, dependent on the energy state of the subconfluent cells, which increased by a factor of two in cells treated with NaN₃. Of interest is that in NaN₃-treated subconfluent cells, the mobility of HEDAF was not significantly different from that of the (Na⁺,K⁺)ATPase.

The mobility of the (Na⁺,K⁺)ATPase on the basolateral surface of confluent cells was not significantly different from that on the apical surface of subconfluent cells. However, it was not possible to determine whether tearing the cell layer to expose the basolateral surface altered the mobility characteristics of the (Na⁺,K⁺)ATPase. Nevertheless, it is unlikely that there was a major alteration since U and Evans-Layng reported the same distribution of (Na⁺,K⁺)ATPase in their preparations and demonstrated that tight junctions and asymmetry in distribution of the (Na⁺,K⁺)ATPase were preserved even when samples were processed as detached sheets.

Discussion

The results presented in this paper indicate that the (Na⁺,K⁺)ATPase is 50% immobilized in both subconfluent and confluent cultures of MDCK cells. The mobile fraction shows a relatively high diffusion constant, which suggests that half of the (Na⁺,K⁺)ATPase molecules are freely diffusing in the plasma membrane. Addition of azide increases the rate of diffusion of the mobile fraction but has no effect on the size of the immobile fraction. Mobility measurements with the lipid probe HEDAF demonstrate near-complete FRAP, which is typical of lipids and indicates that the probe has a higher mobility in confluent cells and a diffusion coefficient that approaches that of the (Na⁺,K⁺)ATPase in subconfluent cells.

Our results can be compared with those reported by Dragsten et al. (11). The diffusion coefficient that we measured for HEDAF was not significantly different from their reported value of $4.2 \pm 1.8 \times 10^{-9}$ cm²/s for the same probe. In their study, however, wheat germ agglutinin receptors appeared to be completely immobile. Unfortunately, wheat germ agglu-

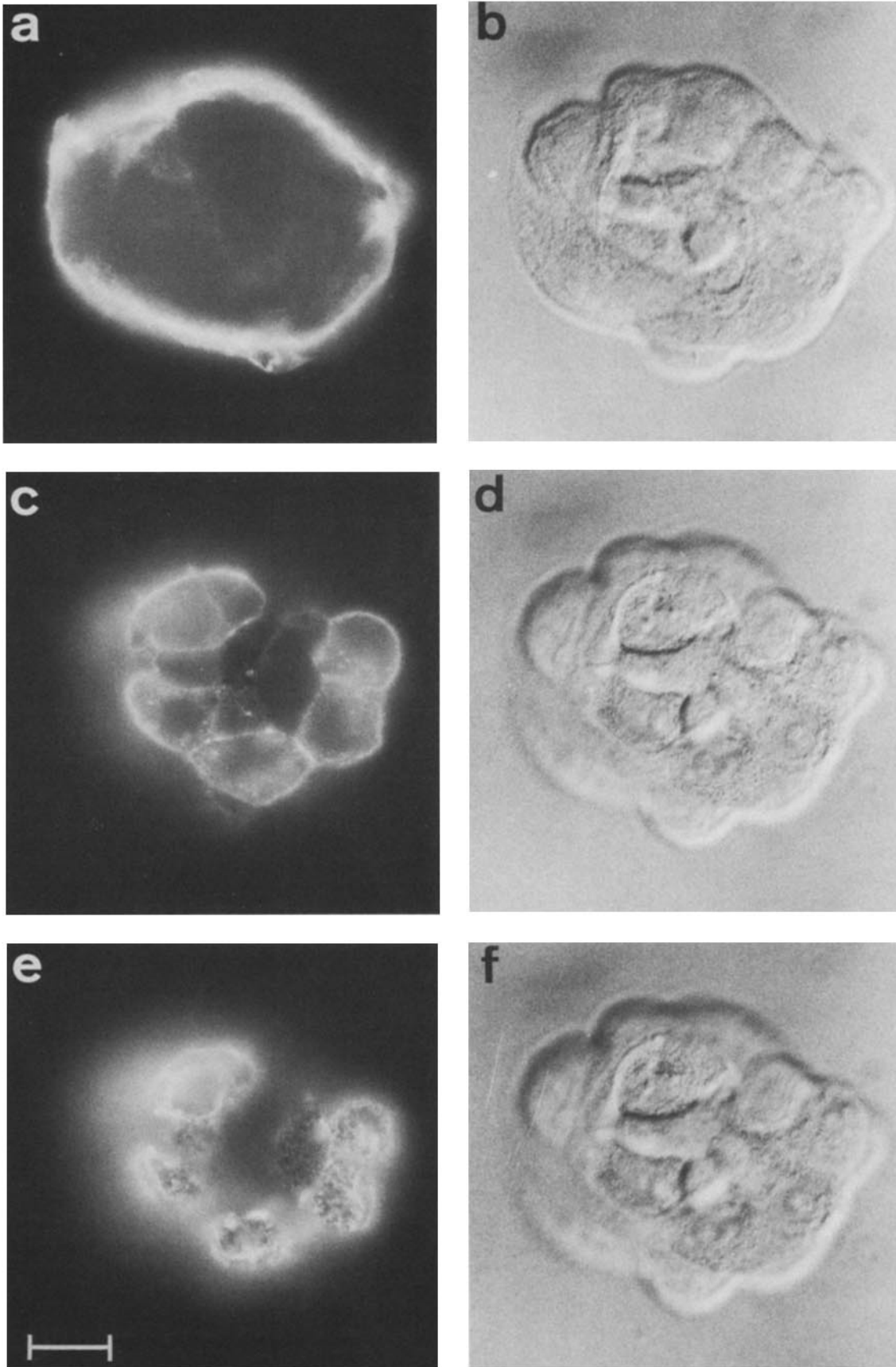


Figure 2. Immunofluorescence localization of the $(\text{Na}^+, \text{K}^+)\text{ATPase}$ by rabbit anti-dog $(\text{Na}^+, \text{K}^+)\text{ATPase}$ on the apical surfaces subconfluent islands of MDCK cells. Different focal planes of observation show that peripheral cells are labeled most strongly in *a*, *c*, and *e*. Corresponding regions using Nomarski optics are shown in *b*, *d*, and *f*. Bar, 10 μm .

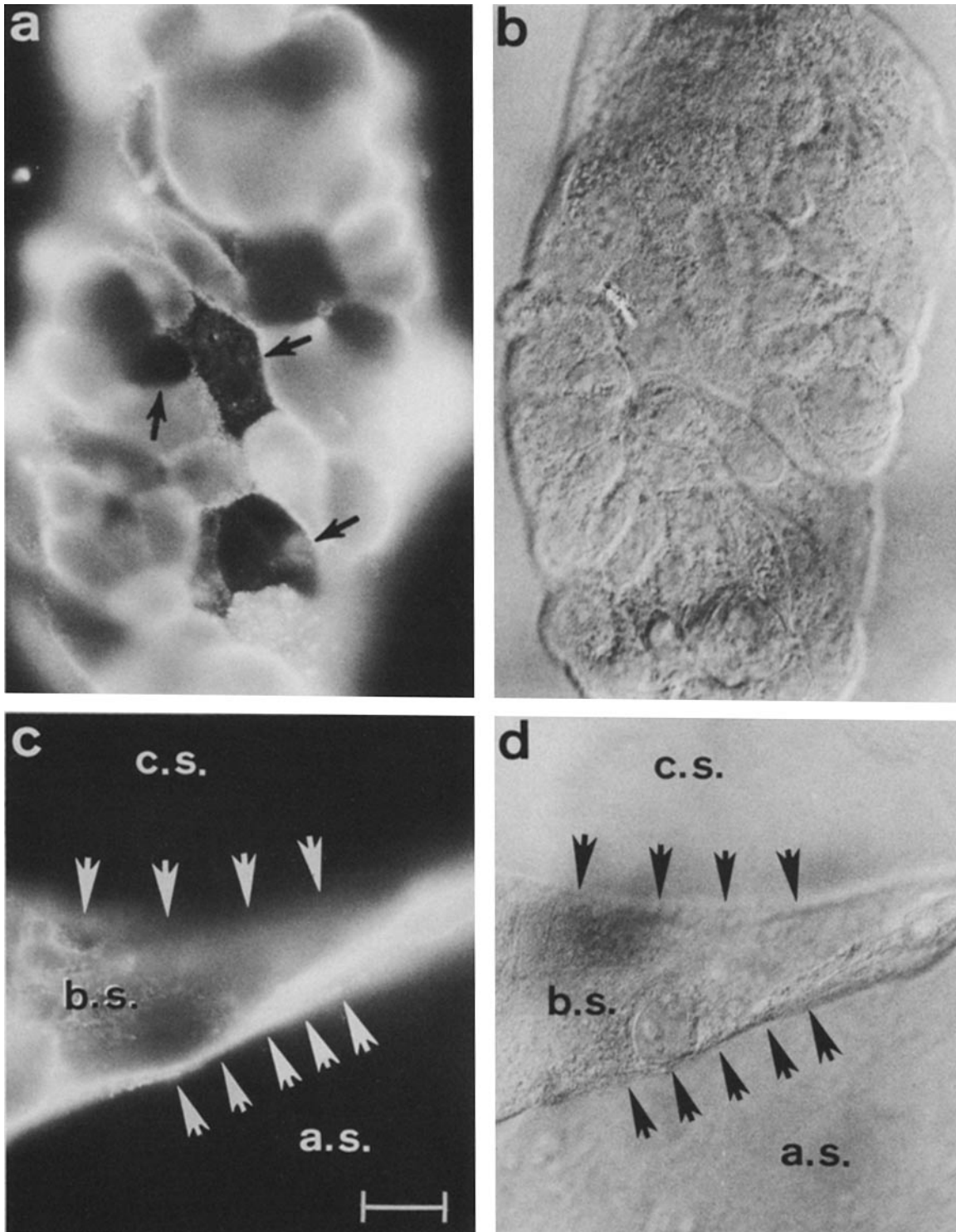


Figure 3. Heterogeneity in immunofluorescence localization of the $(\text{Na}^+, \text{K}^+)\text{ATPase}$ by rabbit anti-dog $(\text{Na}^+, \text{K}^+)\text{ATPase}$ on the apical surfaces of larger subconfluent regions of MDCK cells. A large cell island is shown in *a* and *b*. Arrows indicate cells that did not label strongly with antibody. The torn edge of an extended confluent region is shown in *c* and *d*. Area between arrowheads indicates an edge, folded back demonstrating that the basolateral surface (*b.s.*) is very brightly stained relative to the apical surface (*a.s.*). Region of the coverslip is denoted by *c.s.* Bar, $10 \mu\text{m}$.

tinin lacks specificity in that it recognizes N-acetylglucosamine, or sialic acid residues of a variety of glycolipids and glycoproteins and, therefore, potentially can bind to all membrane components that contain these residues. In addition, since it is a multivalent ligand, it may cross-link mobile

proteins or lipids and immobilize them or induce cytoskeletal associations by cross-linking previously mobile proteins (2, 29). Consequently, it is difficult to determine from such results whether any particular species of membrane constituent recognized by wheat germ agglutinin is indeed immobilized in

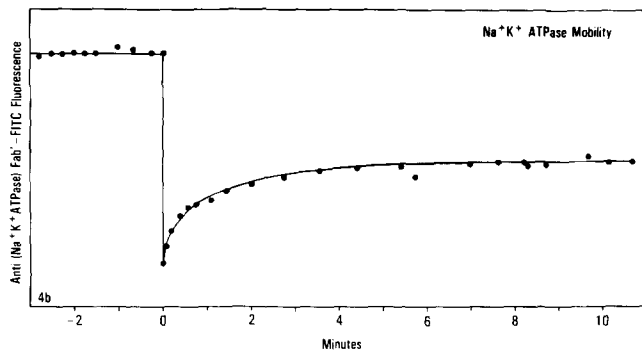


Figure 4. FRAP of FITC-conjugated rabbit anti-dog (Na^+, K^+)ATPase Fab' fragments specifically labeling the apical surfaces of subconfluent culture MDCK cells. Fluorescence was excited in a 2.5- μm -diam circular spot on the cell surface by a focused He-Cd laser microbeam ($\lambda_{\text{ex}} = 451 \text{ nm}$) and measured through microscopic optics by photon counting for several seconds at 15- to 45-s intervals. From such curves, the percent recovery, R , and half time of recovery, $t_{1/2}$, were used to determine the mobile fraction and lateral diffusion constant of the (Na^+, K^+)ATPase.

the native state of the cell.

Since the (Na^+, K^+)ATPase is itself a wheat germ binding protein (30), our study using a monovalent anti-(Na^+, K^+)ATPase antibody label indicates that at least one membrane wheat germ agglutinin binding protein is not completely immobile. The sizeable (50%) mobile fraction of the (Na^+, K^+)ATPase exhibited a diffusion constant of $2\text{--}5 \times 10^{-10} \text{ cm}^2/\text{s}$, which is only a factor of 5–10 smaller than rhodopsin, the most rapidly diffusing integral membrane protein yet measured. It must be cautioned, however, that the measurements on confluent cells involved tearing of the cell layer to gain access to the basolateral surface. These measurements, therefore, may not be representative of the true mobility of the protein in the basolateral membrane of the intact cell layer if some metabolic or regulatory functions of the cell had been sufficiently perturbed. However, as stated in the Results section, it is unlikely that major alterations have occurred based on the results of U and Evans-Layng (40).

What, then, could be the functional significance of the observed immobilization of a major fraction of an integral membrane transport protein such as the (Na^+, K^+)ATPase? Since the protein has been fully purified and functionally reconstituted, it is clear that it can be active without being immobilized. Antibodies directed to its large cytoplasmic domain, however, can be inhibitory (21). One possibility is that its immobilization might be due to binding of regulatory proteins. Alternatively, the immobilization could be related to the transfer of the (Na^+, K^+)ATPase to and from hypothetical plasma membrane–cytoskeletal “loading docks” where it would be delivered or received from the endomembrane system. The latter hypothesis receives support from recent experiments suggest that the cytoskeleton is involved in polarized delivery of membrane proteins to the plasma membrane from the Golgi apparatus (19, 22, 36), and in their endocytosis via the coated pit mechanism (4, 37).

Our results are, however, of significance with respect to understanding how a cell establishes and maintains an asymmetric distribution of its plasma membrane protein components (1, 3, 13). There are at least three mechanisms for maintaining such polarity. These are (a) immobilization; (b)

barriers to diffusion such as tight junctions or closely spaced and immobilized proteins on or in the membrane (12); (c) polarized insertion and rapid endocytosis (5). The first mechanism may be mediated by association of plasma membrane proteins with a polarized network of cytoskeletal proteins (2, 19). Such an association may be involved in immobilizing half of the (Na^+, K^+)ATPase to the basolateral surface of the cell. It must be noted, however, that there is no direct evidence for such an association and that lateral immobilization in microscopic domains of the order of the FRAP measuring beam dimension do not preclude unrestricted lateral protein mobility in smaller lateral domains. Support for this view is derived from experiments that show that certain membrane proteins are restricted in their lateral diffusion but are rotationally unconstrained in the plane of the membrane (9, 33).

This latter dichotomy in membrane mobilities has led to proposal of molecular barriers to lateral diffusion (12, 28, 38). Such barriers in the form of molecular networks distributed in the plane of the membrane or localized to tight junctions could serve as the basis for the second mechanism for maintaining the polarity of the mobile fraction of the (Na^+, K^+)ATPase.

The third alternative relies on continuous and spatially asymmetric vesicular delivery of the (Na^+, K^+)ATPase to the plasma membrane. Such delivery would be followed by rapid internalization. In this case, a spatial gradient of the ATPase could be maintained without the necessity for a barrier to diffusion or for anchorage. This third mechanism for maintaining polarity has been recently discussed by Almers and Stirling (1). They have presented an expression $\lambda = (TD)^{1/2}$, which estimates the mean diffusion distance, λ , of a laterally mobile molecule having a diffusion constant, D , inserted in the plasma membrane at time $t = 0$, and removed at $t = T$ by an internalization event. If we assume that insertion occurs at a lateral domain of the plasma membrane, close to the *trans*-Golgi apparatus as has been demonstrated for viral proteins in infected MDCK cells (36), then endocytosis would have to occur by the time the molecules would diffuse to the region of the tight junctions where the abrupt change in surface density of the (Na^+, K^+)ATPase occurs (40).

An estimate for an upper limit for this distance would assume delivery of the (Na^+, K^+)ATPase to lateral membrane most proximal to the basal surface. Morphometric determination of apical surface area at $214 \mu\text{m}^2$ and total surface area of $1,840 \mu\text{m}^2$ (31) suggests that deposition of protein on the basolateral edge of a cuboidal cell would interpose a maximum diffusion distance of $\sim 24 \mu\text{m}$ to the apical–lateral edge. Given this distance the maximum surface residency time calculated from the expression given above is $\sim 3 \text{ h}$. If, however, delivery of the (Na^+, K^+)ATPase is closer to the tight junctions as the relatively uniform distribution of the (Na^+, K^+)ATPase over the basolateral domain (40) suggests, then residency times would have to be much shorter. A $1\text{-}\mu\text{m}$ diffusion distance would require a residency time of only 20 s.

Several observations indicate the existence of asymmetric insertion and removal of membrane proteins in MDCK cells. Rindler et al. (36) have shown that at 37°C and 15 min after viral G protein is observed in the Golgi apparatus of virally infected MDCK cells, it is inserted at any place, along the apical to basal dimension in the lateral plasma membrane,

which include regions adjacent to the tight junctions. The protein achieves a relatively uniform basolateral distribution similar to that of the (Na⁺,K⁺)ATPase (40) by 45 min after observation in the Golgi apparatus. Similar patterns and kinetics for the G protein have also been reported by Matlin and Simons (24) and Pfeiffer et al. (31). By inference, delivery of this protein must be balanced by an equivalent rate of removal by endocytosis. Equivalent rates have been demonstrated by Louvard (22) for the endocytosis of aminopeptidase from the apical surface of MDCK cells.

Although these results are for proteins other than the (Na⁺,K⁺)ATPase (6), they indicate that the cells are capable of such turnover. Indeed recent evidence suggests similar processes in the case of the (Na⁺,K⁺)ATPase. Additional evidence in support of this view has come from our observation that cells on slides kept at room temperature for lateral mobility studies for longer than 45 min to 1 h developed visible internal fluorescence that could be blocked by the metabolic inhibitor NaN₃. Such internal fluorescence might, therefore, be a manifestation of endocytosis of the (Na⁺,K⁺)ATPase-Fab' complex and would therefore not differ significantly in kinetics to that inferred for the G protein system. Thus, if ATPase is delivered to the lateral membrane in regions close to the tight junction (~1-5 μM) there would be insufficient time for its removal by endocytosis to prevent its diffusion across the region delineated by the tight junctions.

In summary, our results suggest that the immobilization of the (Na⁺,K⁺)ATPase can be only partially responsible for maintenance of the polarity of its distribution in MDCK cells. The uniformity of its distribution in the lateral domain, the fidelity of its exclusion from the apical domain, and the incompatibility of its rapid diffusion with the insertion and rapid removal mechanism suggest that localized restricted diffusion at tight junctions (12, 41) is probably the most likely mechanism for maintaining the polarity of the mobile fraction of the (Na⁺,K⁺)ATPase. Clearly many questions still remain about the relationship between vesicular membrane flow and lateral control of membrane proteins. These will provide interesting areas for further study.

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