Analysis of Na⁺,K⁺-ATPase Motion and Incorporation into the Plasma Membrane in Response to G Protein–coupled Receptor Signals in Living Cells^V

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Dopamine (DA) increases Na⁺,K⁺-ATPase activity in lung alveolar epithelial cells. This effect is associated with an increase in Na⁺,K⁺-ATPase molecules within the plasma membrane (Ridge *et al.*, 2002). Analysis of Na⁺,K⁺-ATPase motion was performed in real-time in alveolar cells stably expressing Na⁺,K⁺-ATPase molecules carrying a fluorescent tag (green fluorescent protein) in the α -subunit. The data demonstrate a distinct (random walk) pattern of basal movement of Na⁺,K⁺-ATPase-containing vesicles in nontreated cells. DA increased the directional movement (by 3.5 fold) of the vesicles and an increase in their velocity (by 25%) that consequently promoted the incorporation of vesicles into the plasma membrane. The movement of Na⁺,K⁺-ATPase-containing vesicles and incorporation into the plasma membrane were microtubule dependent, and disruption of this network perturbed vesicle motion toward the plasma membrane and prevented the increase in the Na⁺,K⁺-ATPase activity induced by DA. Thus, recruitment of new Na⁺,K⁺-ATPase molecules into the plasma membrane appears to be a major mechanism by which dopamine increases total cell Na⁺,K⁺-ATPase activity.

INTRODUCTION

In polarized epithelia the Na⁺,K⁺-ATPase is located within the basolateral domain of the cell and drives vectorial transport of sodium (Rodriguez-Boulant and Nelson, 1989; Skou and Esmann, 1992; Caplan, 1997; Dunbar and Caplan, 2001). This function is performed in combination with the activity of sodium channels located at the apical domain of the cell. Short-term regulation of Na⁺,K⁺-ATPase activity is a com-

plex process that requires the integration of multiple intracellular signaling networks that in most cases are cell specific (Bertorello and Katz, 1993; Therien and Blostein, 2000; Feraille and Doucet, 2001; Dunbar and Caplan, 2001). The action of these networks ultimately results in activation of protein kinases and phosphorylation of the Na⁺,K⁺-ATPase catalytic α -subunit (Therien and Blostein, 2000). Depending on the type and isoform of the kinase their action could lead to a decrease or increase in cell Na⁺,K⁺-ATPase activity (Carranza *et al.*, 1998; Efendiev *et al.*, 1999, 2000; Ridge *et al.*, 2002). However, phosphorylation may not affect the intrinsic properties of the Na⁺,K⁺-ATPase (i.e., catalytic activity) but their subcellular distribution. For example, in renal epithelial cells inhibition of Na⁺,K⁺-ATPase activity by catecholamines or phorbol esters is mediated by phosphorylation of the catalytic α -subunit, and this event does not affect the catalytic properties of the enzyme while in the plasma membrane but constitutes the triggering signal for its endocytosis into endosomes via a clathrin vesicle-dependent mechanism (Chibalin *et al.*, 1997, 1999; Yudowski *et al.*, 2000;

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Abbreviations used: DA, dopamine; MT, microtubules; NOC, nocodazole; TX, taxol; LAT, latrunculin B; GFP, green fluorescent protein; GFP-NK α_1 , green fluorescent protein-tagged Na⁺,K⁺-ATPase α_1 -subunit.

Efendiev *et al.*, 2002). By contrast, in lung alveolar epithelial cells isoproterenol or DA increase Na^+ , K^+ -ATPase activity and this effect is associated with an increased recruitment of active Na^+ , K^+ -ATPase molecules to the plasma membrane (Bertorello *et al.*, 1999; Lecuona *et al.*, 2000; Ridge *et al.*, 2002). The molecular mechanisms responsible for the increase in Na^+ , K^+ -ATPase activity and the translocation from intracellular compartments to the plasma membrane are not yet completely understood.

In vitro studies have suggested that the Na^+ , K^+ -ATPase activity in intact cells is operating at one third of its maximal capacity (Skou and Esmann, 1992). Thus, this reserve capacity of the enzyme would indicate several possible ways of regulation in intact cells: 1) changes in the number of units at the plasma membrane; 2) changes in the catalytic property of enzymes already present at the plasma membrane; or both.

Thus, the aim of this study was to determine whether DA signals increase the directional motion of Na^+ , K^+ -ATPase-containing vesicles and whether this motion leads to incorporation of new Na^+ , K^+ -ATPase molecules at the plasma membrane and increases in Na^+ , K^+ -ATPase activity.

MATERIALS AND METHODS

Reagents

Polyclonal antibody against green fluorescent protein (GFP) was obtained from Clontech. Rat mAb against α -tubulin was a gift from J.V. Kilmartin (Laboratory of Molecular Biology, Cambridge, UK). Latrunculin B was obtained from Calbiochem, and nocodazole, taxol, and dopamine were purchased from Sigma. HBSS was from Life Technologies (Gaithersburg, MD). The introduction of a GFP tag at the N-terminus of Na^+ , K^+ -ATPase α_1 -subunit was performed as previously described. (Cotta Doné *et al.*, 2002). A549 cells were obtained from American Type Culture Collection (Manassas, VA). This cell line retains several of the characteristics of rat alveolar type-II cells. The effect of dopamine on Na^+ , K^+ -ATPase activity as well as the signaling mechanisms involved is similar to type-II cells isolated from rat lung (Guerrero *et al.*, 2001; Ridge *et al.*, 2002). Selection of stable clones of A549 cells expressing the GFP-tagged rat Na^+ , K^+ -ATPase α_1 -subunit was performed as described previously (Pedemonte *et al.*, 1997; Ogimoto *et al.*, 2000). LysoTracker Green DND-26 (LTG) was purchased from Molecular Probes (Eugene, OR).

Determination of Na^+ , K^+ -ATPase Activity

Na^+ , K^+ -ATPase activity was determined in A549 cells as the rate of ouabain-sensitive [$^{86}\text{Rb}^+$]RbCl transport (Pedemonte *et al.*, 1997) in cells that had been previously incubated with 1 μM DA (5 min) before the assay.

Immunofluorescence Microscopy

A549 cells were fixed for triple immunostaining of actin, MTs, and GFP-NK α_1 vesicles as described (Tournebise *et al.*, 2000). Briefly, A549 cells were fixed in 3–4% paraformaldehyde and 0.1% glutaraldehyde plus 0.5% Triton X-100 in BRB80 buffer (80 mM PIPES, 1 mM MgCl_2 , 1 mM EGTA, pH 6.8). Cells were incubated with a 1:30 dilution of rat monoclonal antitubulin antibodies and a 1:50 dilution of rhodamine-labeled phalloidin (Sigma). Anti-rat IgG coupled to Cy5 (Jackson Immuno Research Laboratories, Inc.) was used as secondary antibody. Stained cells were washed and mounted in Aqua-PolyMount medium (Polyscience, Inc.). Images were acquired with an inverted microscope (Nikon) and were further processed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Na^+ , K^+ -ATPase Imaging in Live Cells

A549 cells expressing the GFP-tagged Na^+ , K^+ -ATPase α_1 -subunit (GFP-NK α_1) were grown on glass coverslips attached to 35-mm plastic dishes with a hole in the center of the dish. Experiments were performed 36 h after plating. Digital fluorescence images were acquired as a time-lapse series with a Photometrics CH 350 cooled CCD camera (Tektronics 512 $\times$ 512 black-thinned CCD array). Cells were kept at 37°C during the microscopic observation, and the temperature under the objective was measured before and after experiment. Each series contained at least 300 images taken at 1-s interval with HQ-GFP filter (Chroma Optical, Brattleboro, VT). Movement of vesicles for each experimental condition was established by acquiring at least 50 images before addition of the agonist. DA was added to the dish during the time-lapse series. Cell cultures were treated with the oxygen-depleting preparation, Oxyrase (Oxyrase, Inc., Ashland, OH), to reduce photodamage and photobleaching. Images (16-bit) were processed and rescaled with Metamorph software (Universal Imaging Corp., West Chester, PA), and 8-bit images were prepared for presentation with Adobe Photoshop. The Metamorph software was also used to calculate the position and velocity of vesicles and to generate traces for vesicle motion.

Miscellaneous

Plasma membranes were prepared using an identical procedure as described before (Lecuona *et al.*, 2000). Proteins were quantitated (Bradford, 1976) and analyzed by SDS-PAGE (Laemmli, 1970). Western blot was performed as described before (Ridge *et al.*, 2002) using a commercial luminescence kit (Amersham Pharmacia Biotech).

Statistics

Statistical analysis of the data was performed with a nonpaired Student's *t* test; *p* values < 0.05 were considered significant.

RESULTS

Incorporation of Na^+ , K^+ -ATPase-containing Vesicles into the Plasma Membrane

Because incorporation of Na^+ , K^+ -ATPase molecules within the plasma membrane appears to be responsible for increases in Na^+ , K^+ -ATPase activity in response to G protein-coupled receptor signals (Bertorello *et al.*, 1999; Efendiev *et al.*, 2000; Ridge *et al.*, 2002), we performed experiments to evaluate the mechanisms that govern the motion of Na^+ , K^+ -ATPase-containing vesicles. Experiments were performed in A549 cell (human lung origin with characteristics of alveolar Type II cells) expressing stably the rodent Na^+ , K^+ -ATPase α_1 -subunit tagged with GFP (GFP-NK α_1). The Na^+ , K^+ -ATPase is located at the basolateral domain of polar alveolar epithelial cell. Because in this study the A549 cells were not grown on permeable support (lack polarity and thereby basolateral/apical organization), the Na^+ , K^+ -ATPase was randomly distributed along the plasma membrane. The GFP-NK α_1 signal was detected along the plasma membrane and as punctate structures within the cytosol (Figure 1A). Analysis of GFP-NK α_1 revealed two distinct structures of 0.7–0.9- μm diameter, mostly concentrated in the perinuclear region and vesicles of 0.2–0.3- μm diameter throughout the cytoplasm and near the plasma membrane (consistent with the size of individual clathrin vesicles; Anderson *et al.*, 1978; Gaidarov *et al.*, 1999). Total cell Na^+ , K^+ -ATPase activity was not different in A549 cells expressing stably the GFP-NK α_1 or the nontagged isoform (Figure 1B). Similarly,

Figure 1. Incorporation of GFP-NK α_1 into the plasma membrane. (A) Intrinsic GFP-NK α_1 fluorescence in A549 cells grown on coverslips. (B) Na⁺,K⁺-ATPase activity in the presence (DA) or absence (V) of 1 μ M DA (5 min at 23°C) was determined in A549 cells expressing either the GFP-NK α_1 (green bars) or the nontagged (black bars) isoform. Each bar represents the mean $\pm$ SE of four experiments performed in triplicate. (C) GFP-NK α_1 abundance in the basolateral plasma membrane prepared from A549 cells previously treated with (DA) or without (V) 1 μ M DA (5 min at 23°C). Na⁺,K⁺-ATPase was detected by Western blot (Laemmli, 1970) with a GFP antibody or (in the same membrane after stripings) with an α -subunit antibody. Equal amount of protein (Bradford, 1976) was analyzed in each lane. (D) Top panel: Low magnification image of the live A549 cell stably expressing GFP-NK α_1 . Lower panels: Enlarged time-lapse sequence of region boxed in top panel. First image was acquired 27 s before addition of DA (indicated by the filled arrowhead). Progression of GFP-NK α_1 to the plasma membrane is denoted by the arrowheads. Numbers in the right upper corners indicate time in seconds; scale bar, 5 μ m.

DA stimulated Na⁺,K⁺-ATPase activity to the same magnitude in both groups (Figure 1B), and the GFP-NK α_1 abundance (detected with either GFP- or α_1 -subunit antibody) in

the plasma membrane was proportionally increased by DA in both groups (Figure 1C). Expression of the endogenous Na⁺,K⁺-ATPase (untagged) was negligible as previously demonstrated (Chibalin *et al.*, 1998). Sequential time-lapse images after addition of DA captured the motion of GFP-NK α_1 and their rapid incorporation into the plasma membrane (Figure 1D). These events occurred within 60 s (average) after DA stimulation. Longer time analysis demonstrated that incorporation of additional GFP-NK α_1 took place in the same area of the cell, most frequently in zones of cell-to-cell contacts, whereas no fusion was detected in other randomly chosen areas of the plasma membrane within the same cell.

Analysis of Na⁺,K⁺-ATPase-containing Vesicles Motion

Under basal nonstimulated conditions GFP-NK α_1 -containing vesicles move constantly, following a "biased random-walk" pattern within the cytoplasm. Projection analysis—merge stacks of equal numbers of successive frames before and after addition of the agonist demonstrate that DA stimulates long-range linear movement of the vesicles (Figure 2A). Using an automated tracking program, we determined the original position of randomly chosen vesicles and analyzed their displacement before and after the addition of DA (Figure 2B). Trajectories of GFP-NK α_1 examined during 100 s before and after the addition of DA showed qualitative (unidirectional movement) and quantitative (about fourfold larger extent of the movement) changes induced by the agonist. A significant displacement of vesicles from their point of origin could be also visualized for longer time after addition of DA (Figure 2C). Not all vesicles change the character of the motion simultaneously after addition of DA: although some vesicles responded faster and changed their random-walk displacement to unidirectional within 50–100 s after DA addition (red, purple, and green lines), others appeared to respond after 5–8-min stimulation and the characteristics of their motion appeared to be an increase in the random-walk pattern (blue and light blue lines). It can also be appreciated from this analysis that 44% $\pm$ 6, n = 6 cells (~20 vesicles analyzed per cell) of the vesicles increased their linear motion in response to DA. An increase in vesicle unidirectional displacement >80% from basal (before DA stimulation) was the criteria considered as an increased response to DA. Statistical analysis of GFP-NK α_1 that showed unidirectional displacement in response to DA, indicated an increase (fourfold) in the extension of the motion and also an accelerated velocity of the movement by ~25% (5.2 vs. 4.2 μ m/min in nontreated cell; Figure 2D). The unidirectional motion of GFP-NK α_1 occurred mostly in vesicles localized at the cell periphery rather than in vesicles located in the perinuclear area. The action of DA was specific for GFP-NK α_1 -containing vesicles, as acidic organelles and lysosomes labeled with LTG (Figure 2E), exhibited neither significant unidirectional movements (<7% $\pm$ 2, n = 4 cells; 16 vesicles examined per cell), nor changes in their velocity and displacement (Figure 2F) upon addition of 1 μ M DA. Further proof of specificity was obtained in cells treated with a sodium ionophore. Addition of monensin increases the Na⁺,K⁺-ATPase catalytic activity in epithelial cells (Efendiev *et al.*, 2002) without affecting the incorporation of new molecules into the plasma membrane. Accordingly, displacement analysis did not demonstrate any changes upon addition of monen-

Figure 2. Motion of GFP-NK α_1 . (A) Projection image was generated by merging successive frames of a stack, collected at 3-s intervals. If the object displays motion its position shifts accordingly in the next frame and on the merge image, it creates a trajectory of the motion (linear if it moves directionally or nonlinear if random-walk displacement occurs). Each projection contains 50 frames before and after addition of DA. Arrows show the starting point. Scale bar, 5 μm . (B) Vesicles were tracked during 100 s before and after addition of DA. Each of the paired tracks (before and after) represents the motion of the same vesicle. Circles on the right side show the average of displacement corresponding to several vesicles. (C) Life histories of the vesicle displacement over time before and after addition of DA. Each line represents a different vesicle. (D) Histograms of velocity and displacement of GFP-NK α_1 (average) before (■) and after (□) addition of DA. (E) Life histories of the LTG vesicles displacement over time before and after addition of DA. Each line represents a different vesicle. (F) Histograms of velocity and displacement of LTG vesicles (average) before (■) and after (□) addition of DA.

sin (before: 2.76 ± 0.33 and after monensin: 2.65 ± 0.27 , $n = 3$ cells; 16 vesicles analyzed per cell).

Motion of Na⁺,K⁺-ATPase-containing Vesicles Depends on the Integrity and Dynamics of the Actin-Microtubule Cytoskeleton

Motion of intracellular organelles occurs along the actin-microtubule network (Rogers and Gelfand, 1998; Nielsen *et al.*, 1999). The long-range linear (directional) movements of GFP-NK α_1 vesicles toward the periphery of the cell induced by DA suggest a microtubule (MT)-based process. In the following experiments, manipulation of the cell cytoskeleton with different agents has been limited to the extent of not affecting dramatically the cell structure that could confound the interpretation of the results, and this was confirmed by confocal microscopy.

Incubation of A549 cells with nocodazole (NOC) significantly disrupted the MT array without affecting the actin network (Figure 3A, compare with nontreated cells in Figure 5A) and also promoted the redistribution of GFP-NK α_1 vesicles from the periphery to the perinuclear area of the cells. Projection analysis showed that NOC abolished the random-walk motion of GFP-NK α_1 vesicles and the unidirectional

displacement induced by DA (Figure 3B, also compare with Figure 2A). The proportion of moving GFP-NK α_1 vesicles in response to DA was significantly decreased (4.2 ± 2 , $n = 3$ cells, ~ 20 vesicles analyzed per cell). Motion analysis of those vesicles that did move in response to DA for a longer period of time (600 s) also illustrates the lower amplitude of their displacement after addition of DA (Figure 3C, compare with Figure 2C). Statistical analysis of those vesicles demonstrated a significant increase in velocity and displacement induced by DA (Figure 3D). However, because motion occurred in a small number of GFP-NK α_1 vesicles, it was not sufficient to promote an increase in Na⁺,K⁺-ATPase activity (Figure 3E) to the same magnitude as in nontreated cells (without NOC).

Taxol (TX) caused the disruption of the radial array of MT and redistribution of short microtubules from the center to peripheral regions of the cell (Figure 4A, compare with nontreated cells in Figure 5A). TX treatment did not affect the basal (instantaneous) velocity of vesicle movement (compare Figure 4D, left panel, ■ with Figure 2D, left panel, ■), whereas it did change the amplitude of the random-walk movement by $\sim 35\%$ and partially reduced the number of moving vesicles in response to DA (22.1 ± 2 , $n = 4$ cells,

Figure 3. Effect of MT depolymerization on GFP-NK α_1 motion. (A) Immunostaining of the actin network and MT after nocodazole (NOC) treatment (1 $\mu\text{g}/\text{ml}$ for 1 h at 37°C). Red color represents the actin cytoskeleton, green, MT; and blue, GFP-NK α_1 in the merged image. Scale bar, 10 μm . (B) Projection images generated as described in Figure 2B. Scale bar, 5 μm . (C) Life histories of the vesicle displacement in cell treated with NOC before and after DA stimulation. (D) Histograms of velocity and displacement of GFP-NK α_1 (average) in NOC-treated cells before (■) and after (□) addition of DA. The histograms were created as described in Figure 2E. (E) Changes in Na⁺, K⁺-ATPase activity was examined in A549 cell previously treated with NOC (as in A). Incubation with DA 1 μM or vehicle (Hanks' medium) was performed during 5 min at 23°C. Enzyme activity was expressed as nmol Rb/mg prot/min. Data represents the mean + SE of five experiments in triplicate determinations.

~20 vesicles analyzed per cell). In addition, DA increased the displacement of those vesicles, although to a much lesser extent than in cells not exposed to TX and after a lag time of >5 min (Figure 4C). Although DA increased the distance of the movement (Figure 4D, right panel, □), it did not increase their velocity (Figure 4D, left panel). However, it was noticed that about an equal proportion of vesicles moved in both directions—toward cell periphery and toward cell center, but most of them did not reach a plasma membrane. Therefore, in TX-treated cells DA stimulation was not sufficient to increase Na⁺,K⁺-ATPase activity to the level of nontreated cells (Figure 4E).

The role of the actin cytoskeleton in this process was evaluated by pretreating the cells with latrunculin B (LAT). The low concentration of LAT promoted the loss of actin filaments within the cell; only cortical fibers remained, and the cells adopted a round shape (Figure 5A). The MT network was not affected by this treatment. The motion of GFP-NK α_1 under nonstimulated condition was not impaired, but rather slightly stimulated (Figure 5B, right panel, ■, compared with Figure 2D, right panel, ■). LAT did not affect the proportion of vesicles that experience motion in response to DA (DA without LAT: 44% $\pm$ 6, n = 6 vs. DA + LAT: 43% $\pm$ 4, n = 6). In addition, DA did not increase the

Figure 4. Effect of MT stabilization on GFP-NK α_1 motion. (A) Immunostaining of actin and MT after taxol (TX) treatment (5 $\mu\text{g}/\text{ml}$ for 2 h at 37°C). Red color represents actin cytoskeleton, green, MT; blue, GFP-NK α_1 on the merged image. (B) Projection images generated as described in Figure 2B. Scale bar, 5 μm . (C) Life histories of GFP-NK α_1 displacement in cells treated by TX before and after DA stimulation. (D) Histograms of velocity and displacement of GFP-NK α_1 (average) in TX-treated cells before (■) and after (□) addition of DA. The histograms were created as described in Figure 2E. (E) Changes in Na $^+$, K $^+$ -ATPase activity was examined in A549 cell previously treated with TX (as in A). Incubation with DA 1 μM or vehicle (Hanks' medium) was performed during 5 min at 23°C. Enzyme activity was expressed as nmol Rb/mg prot/min. Data represents the mean $\pm$ SE of five experiments in triplicate determinations.

velocity, but significantly increased the amplitude of the random-walk movement (Figure 5B), although to a much lesser magnitude when compared with cells not treated with LAT. Nevertheless, it appears that LAT treatment did not affect the incorporation of Na $^+$, K $^+$ -ATPase molecules into the plasma membrane as DA was able to increase Na $^+$, K $^+$ -ATPase activity similar to levels as in nontreated cells (Figure 5C).

DISCUSSION

This study provides the first evidence of Na $^+$, K $^+$ -ATPase motion in response to G protein-coupled receptor signals in

living cells. The combined tracking of individual Na $^+$, K $^+$ -ATPase-containing vesicles with quantitative analysis of their motion indicates that the intracellular signals regulate the velocity as well as the directional displacement of the vesicles, and also it reflects the importance of the actin-microtubule network during their traffic to and incorporation into the plasma membrane. In addition, this study suggests that motion and incorporation into the plasma membrane constitutes a major mechanism by which hormones, such as dopamine, can increase the Na $^+$, K $^+$ -ATPase activity in intact cells.

Figure 5. Effect of the actin cytoskeleton on GFP-NK α_1 motion. (A) Immunostaining of actin and MT after latrunculin (LAT) treatment (100 nM for 30 min). Red color represents actin, and cyan MT on the merged image. Scale bar, 10 μ m. (B) Histograms of velocity and displacement of GFP-NK α_1 (average) in latrunculin-treated cells before (■) and after (□) addition of DA (bars). The histograms were created as described in Figure 2D. (C) Changes in Na⁺,K⁺-ATPase activity was examined in A549 cell previously treated with latrunculin (as in A). Incubation with DA 1 μ M or vehicle (Hanks' medium) was performed during 5 min at 23°C. Enzyme activity was expressed as nmol Rb/mg prot/min. Data represents the mean $\pm$ SE of five experiments in triplicate determination.

Under basal (nonstimulated) conditions, the movement of GFP-NK α_1 follow a random-walk pattern at speeds of $\sim 4.2 \pm 0.12$ μ m/min, which is within the lower range described for other organelles in the cell (Blocker *et al.*, 1998). The range of nonlinear movements of GFP-NK α_1 was within an average area of ~ 1.2 μ m in diameter. This was twice the area observed for clathrin vesicles (CV) movement in the lower surface and 50% higher for the upper surface of COS-1 cells (Gaidarov *et al.*, 1999). The differences in displacement could be explained by either intrinsic properties of a subpopulation of vesicles carrying Na⁺,K⁺-ATPase molecules or as a phenomena specific for A549 cells, where the actin network is more relaxed than in COS-1 cells. The experiments using LAT suggest the later possibility, because in A549 cells the GFP-NK α_1 experienced a much lower ($\sim 50\%$) increase in mobility (1.2 μ m in nontreated vs. 1.8 μ m after LAT treatment), whereas in COS-1 cells the amplitude of the motion of CV was increased more than twofold after disruption of the actin network (0.6 vs. 1.5 μ m after LAT treatment; Gaidarov *et al.*, 1999).

Incubation of A549 cells with DA (within <100 s) promoted a directional motion of Na⁺,K⁺-ATPase molecules that ultimately led to their incorporation into the plasma membrane. Initially, vesicles located within the cell periphery (not those present in the perinuclear area) responded to DA by their rapid incorporation into the plasma membrane, suggesting that the DA signal (short-term) may not influence the traffic of Na⁺,K⁺-ATPase molecules from the other compartments, such as exit from the Golgi network. However, the displacement of GFP-NK α_1 distant from the plasma membrane occurred later, after a lag of ~ 5 min, and the speed of their movement was also increased in the presence of DA. A portion of these vesicles appears either to fuse later with the plasma membrane, or they remain dis-

playing random-walk movements without any further directional displacement. Conceivably, the early events are directly responsible for the rapid increase in cell Na⁺,K⁺-ATPase activity, whereas the later events could be important for replenishing the endosomes from where the Na⁺,K⁺-ATPase molecules are to be transported to the plasma membrane. Incorporation of Na⁺,K⁺-ATPase molecules at the plasma membrane occurred repeatedly (more than one event over a period of time) at a preferred site within the plasma membrane, whereas other areas observed for longer period of time did not experience any fusion events. These observations suggest the existence of complex cellular factors (i.e., receptor signaling network) responsible for coordinating the receptor response in time and space ("hot spots"). The fact that acidic compartments (endosomes/lysosomes) visualized with LTG did not experience unidirectional motion in response to DA, further indicates the role of endosomal-derived CV as the vehicle for Na⁺,K⁺-ATPase trafficking to the plasma membrane, as well as the specificity of the DA effect. Moreover, increasing the levels of intracellular sodium results in increased Na⁺,K⁺-ATPase catalytic activity and not abundance at the plasma membrane. Consequently, the effect of the sodium ionophore was not associated with any significant change in the random-walk pattern of vesicle motion or their displacement, further indicating a selective response to DA.

It has been suggested that the actin-microtubule network has an important role in the DA-mediated regulation of Na⁺,K⁺-ATPase traffic and activity (Chibalin *et al.*, 1997; Bertorello *et al.*, 1999). Our study demonstrates in A549 cells that motion of vesicles containing Na⁺,K⁺-ATPase molecules was dependent on the structural as well as functional integrity of the microtubule-actin cytoskeleton. NOC induced a redistribution of GFP-NK α_1 to the perinuclear re-

gion of the cells and also decreased significantly (~60%) the spontaneous velocity of the few vesicles that did experience motion. Additionally, NOC significantly decreased the number of vesicles that had responded to DA. However, analysis of the vesicles that did move revealed that NOC increased slightly the velocity of the Na⁺ pumps as compared with untreated cells, but the proportion and magnitude was not sufficient to cause an increase in Na⁺,K⁺-ATPase activity. Under these conditions, vesicle motion in the absence of the MT array probably occurred only along the actin filaments or along a few MT that remained intact despite NOC treatment.

Similarly, in the presence of TX DA did not cause an increase in Na⁺,K⁺-ATPase activity or in α_1 -subunit abundance at the plasma membrane. However, the small proportion of vesicles that moved (~22%) in response to DA did not have a change in their velocity. Detailed analysis of the vesicles' motion demonstrated that a similar number of the vesicles moved in the opposite direction—toward the plasma membrane and backward. Such unusual motion was probably caused by disruption of the radial MT network where the short (stable) MTs become reoriented, and their plus ends were redirected toward the center under TX treatment. We reason that this could provide an explanation for the lack of Na⁺,K⁺-ATPase fusion with the plasma membrane and thereby the lack of increase in Na⁺,K⁺-ATPase activity. Alternatively, because TX impairs MT growth, it may have limited the distance that GFP-NK α_1 could travel along MT.

LAT shifts the equilibrium of the actin network toward nonpolymerized actin (Lyubimova *et al.*, 1997). In our study LAT inhibited the actin filaments without affecting the cortical actin cytoskeleton (see Figure 5A). Because the role of actin networks in vesicle fusion is thought to be particularly important at the cell periphery, it is not expected that this treatment would impair Na⁺,K⁺-ATPase fusion with the plasma membrane and thereby an increase in Na⁺,K⁺-ATPase activity in response to DA. Indeed, LAT treatment did not impair the motion of vesicles in response to DA, on the contrary, it appeared to relax the restriction imposed by the actin web to the movement of vesicles (displacement, $\mu\text{m}/\text{sec}$; vehicle: 1.23 ± 0.03 vs. LAT: 1.78 ± 0.04). The fact that DA was able to increase the displacement of GFP-NK α_1 is consistent with the resulting increase in Na⁺,K⁺-ATPase activity to the same extent as in cells with an intact actin cytoskeleton. The latter probably reflects the existence of actin meshworks that constitutively restricts the movement of vesicles similar to the mechanisms regulating clathrin vesicle movement during endocytosis (Gaidarov *et al.*, 1999) or that in the absence of a stable actin network the MT system could assume the role of delivering the cargo to the site of fusion. An indication that LAT treatment may have also affected the association of Na⁺,K⁺-ATPase vesicles with actin motors is suggested by the findings that the velocity of their movement was not increased in the presence of DA and also supports the hypothesis that the filamentous actin network (or actin motors) might be needed to support the MT plus end traffic in order to achieve a higher velocity and amplitude during directional motion (Rogers and Gelfand, 1998).

Is the unidirectional motion of Na⁺,K⁺-ATPase molecules triggered by DA signals a prerequisite essential for the increase in abundance at the plasma membrane and the con-

sequent increase in activity? The studies in the presence of microtubule-disrupting agents argue in favor of the important role that motion of Na⁺,K⁺-ATPase molecules has for the overall increase in activity. Lack (nocodazole treatment) as well as reorganization (taxol treatment) of microtubules prevented Na⁺,K⁺-ATPase motion and the increase in Na⁺,K⁺-ATPase activity in response to dopamine. Moreover, the studies in the presence of dynamin mutants suggest that formation of clathrin vesicles derived from endosomes (and not preformed clathrin vesicles) were needed to achieve a substantial increase in quantity of molecules at the plasma membrane and thereby increase in Na⁺,K⁺-ATPase activity (unpublished observations). Altogether, these data further support the importance of both, motion and incorporation into plasma membrane of Na⁺,K⁺-ATPase-containing vesicles, as part of the mechanisms by which DA increases Na⁺,K⁺-ATPase activity in epithelial cells.

Administration of DA increased alveolar edema clearance in rodents with normal lungs and during lung injury (Barnard *et al.*, 1999; Saldias *et al.*, 1999). At the cellular level, DA increases Na⁺,K⁺-ATPase activity by mechanisms that include the activation of a signaling network leading to an increase in Na⁺,K⁺-ATPase abundance at the plasma membrane (Ridge *et al.*, 2002). Traffic of Na⁺,K⁺-ATPase molecules from intracellular organelles to the plasma membrane is a relevant mechanism by which catecholamines increase Na⁺,K⁺-ATPase activity and improve alveolar fluid reabsorption. Thus, understanding the cellular mechanisms of this regulation may unveil new target pathways leading to the development of novel therapeutic strategies for the management of pulmonary edema.

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