Quantifying RhoA Facilitated Trafficking of the Epithelial Na\textsuperscript{+} Channel toward the Plasma Membrane with Total Internal Reflection Fluorescence–Fluorescence Recovery after Photobleaching

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The epithelial Na\textsuperscript{+} channel (ENaC) plays a central role in control of epithelial surface hydration and vascular volume. Similar to other ion channels, ENaC activity is set, in part, by its membrane levels. The small G protein RhoA increases ENaC activity by increasing the membrane levels of this channel. We hypothesize that RhoA increases ENaC activity by promoting channel trafficking to the plasma membrane. Few experimental methods are available to directly visualize trafficking of ion channels to the plasma membrane. Here we combine electrophysiology with two complementary imaging methods, total internal reflection fluorescence microscopy and fluorescence recovery after photobleaching, to study the mechanistic basis of RhoA actions on ENaC. Patch clamp results demonstrate that RhoA increases ENaC activity in an additive manner with dominant-negative dynamin. This is consistent with a mechanism of increased ENaC trafficking to the membrane. Direct visualization of ENaC movement near the plasma membrane with total internal reflection fluorescence–fluorescence recovery after photobleaching revealed that RhoA accelerates ENaC trafficking toward the membrane. RhoA-facilitated movement of the channel was sensitive to disrupting the endomembrane system. Moreover, facilitating retrieval decreased ENaC activity but not trafficking toward the membrane. ENaC at the plasma membrane clustered and was laterally immobile suggesting that the cytoskeleton tethers or corrals membrane resident channels or membrane-directed vesicles containing ENaC. Disrupting microtubules but not microfilaments led to reorganization of ENaC clusters and slowed trafficking toward the membrane. The cytoskeleton is an established target for RhoA signaling. We conclude that RhoA, likely through effects on the cytoskeleton, promotes ENaC trafficking to the plasma membrane to increase channel membrane levels and activity.

Ion channels are integral membrane proteins. Regulated trafficking of these proteins to and from the plasma membrane in part controls their activity. This is true for ENaC,\textsuperscript{3} which is a nonvoltage-gated, Na\textsuperscript{+}–selective ion channel. ENaC is localized to the apical plasma membrane of epithelia particularly that lining the distal renal nephron and colon and alveolar spaces (1–3). ENaC activity in this epithelia is limiting for Na\textsuperscript{+} absorption. Thus, ENaC plays an important physiological role in negative feedback control of blood pressure, as well as epithelial surface hydration in the lungs. Indeed, improper regulation and dysfunction of ENaC are causative for some pulmonary and blood pressure disorders (4–6).

Corticosteroids are the primary endocrine modulators of ENaC activity. One mechanism by which these steroids increase ENaC activity is by increasing the levels of this channel in the apical plasma membrane (1–3). We recently reported that the small G protein RhoA similarly increases ENaC activity by elevating membrane levels of the channel (7, 8). Regulation of ion channels, including several types of K\textsuperscript{+} channels (e.g. KCNA, KCNH, and KCNJ), by RhoA and other small G proteins is becoming widely appreciated (9–11). Often, as they do for ENaC (7, 8) and TRPC5 (12), small G proteins in the RhoA family modulate channel activity by influencing their membrane levels. However, important questions and details remain unanswered regarding Rho protein regulation of these channels and in particular RhoA regulation of ENaC. For instance, we do not know the mechanism of action of RhoA on ENaC. Specifically, we do not know whether RhoA promotes ENaC trafficking to the plasma membrane or rather retards retrieval of this channel from the membrane to increase channel activity. Moreover, the protein machinery and cellular structures involved in RhoA actions on ENaC remain undefined.

Rho proteins are GTP-dependent cellular signaling switches (reviewed in Ref. 13). Rho family members localize to the plasma membrane, as well as to intracellular membrane compartments, including endosomes and the Golgi complex. These proteins regulate cytoskeleton organization influencing both microfilaments and microtubules. In addition to or possibly because of effects on the cytoskeleton, Rho proteins play a role.

\textsuperscript{3}The abbreviations used are: ENaC, epithelial Na\textsuperscript{+} channel; TIRF, total internal reflection fluorescence microscopy; FRAP, fluorescence recovery after photobleaching; eYFP, enhanced yellow fluorescent protein; eYPF-M, eYFP membrane marker; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; DN-Dyn, dominant-negative dynamin; m, mouse.
in membrane and vesicle trafficking affecting both endo- and exocytosis. Thus, Rho signaling potentially impinges upon ENaC trafficking to and/or from the membrane via effects on the cytoskeleton.

We test here the hypothesis that RhoA increases ENaC activity by increasing ENaC movement toward the plasma membrane. To test this hypothesis, we combine electrophysiology with a fluorescence imaging strategy joining TIRF microscopy with FRAP to follow movement of ENaC toward the plasma membrane in living cells. We find that RhoA increases ENaC activity by increasing movement of the channel toward the membrane. ENaC at/near the membrane had low lateral mobility and clustered into defined areas, suggesting restricted confinement of the channel at this cellular locale. Microtubules but not microfilaments played an important role in ENaC clustering and movement toward the membrane.

MATERIALS AND METHODS

cDNA Constructs and Cell Culture—All chemicals and materials were purchased from either Fisher, Sigma, Biomol, or Calbiochem unless noted otherwise. CHO and COS-7 cells were maintained with standard culture conditions (Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum, 37 °C, 5% CO2) and transfected using Polymect reagent (Qiagen, Valencia, CA) as described previously (8, 14). The plasmids encoding α-β, and γ-mouse ENaC and NH2-terminal eYFP fusion proteins of these channel subunits have been described previously (15, 16). As noted in these earlier publications, channels consisting of eYFP-tagged subunits exhibit functional behavior indistinguishable from native channels. The plasmid encoding the T635A mutant of γ-mENaC also has been described previously (17). This mutation retards channel retrieval from the plasma membrane by disrupting an endocytic signaling tag embedded within the channel. The expression vector encoding constitutively active (G14V) RhoA was from the UMR cDNA Resource Center. The expression vectors encoding dominant-negative dynamin (Dyn2 K44A-GFP; DN-Dyn) and mouse Nedd4-2 were from M. McNiven (see Ref. 18) and D. Pearce (see Ref. 19). The expression vector encoding the eYFP-M membrane marker was from Clontech. Cells were treated with brefeldin A for 24 h at a concentration of 10 μg/ml. Cells were treated with latrunculin B (20 μM), cytochalasin D (20 μg/ml), and colchicine (500 μM) for 30–40 min. Digiditon (20 μM) was added just prior to experimentation. Cells were fixed with 3.7% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 4 min. Actin was subsequently stained in these cells with Oregon Green 514 phalloidin (Molecular Probes) for 20 min in PBS supplemented with 1% bovine serum albumin.

Electrophysiology—Whole-cell macroscopic current recordings of mENaC expressed in CHO cells were made under voltage clamp conditions using standard methods (8, 14, 17). In brief, current through ENaC was the inward, amiloride-sensitive current. Cells were clamped to a 40-mV holding potential with voltage ramps (500 ms) from 60 down to −100 mV used to elicit current. ENaC activity is the amiloride-sensitive current density at −80 mV. Whole-cell capacitance, on average 8–10 picofarads, was compensated. Series resistances, on average 2–5 megohms, were also compensated.

TIRF Microscopy—Fluorescence emissions from membrane marker (eYFP-M) and eYFP-tagged channel subunits were collected in living cells at room temperature using TIRF (also called evanescent field) microscopy to selectively illuminate the plasma membrane and thus focus on signals from this cellular locale. TIRF generates an evanescent field that declines exponentially with increasing distance from the interface between the cover glass and plasma membrane illuminating only a thin section (≈100 nm) of the cell in contact with the cover glass, including the plasma membrane (20–22). All TIRF experiments were performed in the total internal reflection fluorescence microscopy core facility housed within the Department of Physiology at the University of Texas Health Science Center, San Antonio. We have previously described imaging ENaC containing eYFP-tagged subunits with TIRF microscopy (15, 16). The methods used in this study closely followed these published protocols. In brief, fluorescence emissions from fluorophore-tagged ENaC were collected using an inverted TE2000 microscope with through-the-lens (prismless) TIRF imaging (Nikon). This system was equipped with a vibration isolation system (Technical Manufacturing Corp.) to minimize drift and noise. Samples viewed through a plain Apo TIRF 60× oil immersion and high resolution (1.45 NA) objective. Fluorescence emissions from tagged subunits were collected with the Chroma Technology Corp. 514-nm laser filter set with bandpass emission (Z514BP) by exciting eYFP with an argon ion laser (80 milliwatts) with an acoustic optic tunable filter (Prairie Technologies, Inc.) used to restrict excitation wavelength to 514 nm. In this system, a 514-nm dichroic mirror (Z514rdc) separates the 514/10-nm (Z514/10 BP) and 560/50-nm (HQ560/50m) excitation and emission filters. Fluorescence images were collected and processed with a 16-bit, cooled charge-coupled device camera (Cascade 512F; Roper Scientific Inc.) interfaced to a PC running Metamorph software. This camera uses a front-illuminated EMCCD with on-chip multiplication gain. Images were collected with a 200-ms exposure time immediately before and after photobleaching and every subsequent minute. Images were not binned or filtered with pixel size corresponding to a square of 122 × 122 nm.

FRAP—Fluorophore-tagged channels in and near the plasma membrane and membrane marker were photobleached with TIRF illumination using the argon ion laser (514 nm) at full power for 2 min. With such prolonged photobleaching, this maneuver may be more aptly termed photo-destruction or photolysis of the eYFP tag. Nevertheless, fluorescence emissions from membrane fluorophores were collected under TIRF illumination before and after photobleaching. Laser power, camera gain, and exposure times were constant throughout the course of the experiment except during photobleaching as noted above. All TIRF-FRAP experiments were performed 48–72 h after transfection.
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**RESULTS**

**RhoA Promotes ENaC Trafficking to the Plasma Membrane**—RhoA increases ENaC activity by increasing the membrane levels of this channel (7, 8). Increases in membrane levels result either from increased trafficking of the channel to the plasma membrane or, alternatively, from a decrease in retrieval of the channel from this membrane. Retrieval of ENaC, in part, involves the action of dynamin (17, 24). To distinguish between these two possible mechanisms of action, we tested whether RhoA actions on ENaC were additive with channel retrieval disrupted by expressing dominant-negative dynamin and channels resistant to retrieval. Fig. 1A shows a summary graph of ENaC activity in voltage-clamped CHO cells expressing the channel alone and in the presence of constitutively active (G14V) RhoA and DN-Dyn alone and together. Overexpression of either RhoAG14V or DN-Dyn alone with ENaC significantly increases channel activity. Co-expressing RhoAG14V plus DN-Dyn together with ENaC had additive effects significantly increasing channel activity above that for either alone. This finding is consistent with RhoA increasing ENaC activity through a mechanism independent of dynamin possibly involving increased channel trafficking to the membrane.

The T635A substitution in the γ-subunit of mENaC impairs channel retrieval (17). As apparent in Fig. 1B, ENaC containing this substitution has greater activity compared with channels containing all wild-type subunits. Co-expressing RhoAG14V with T635A-substituted ENaC had an additive effect further increasing channel activity above either alone. This finding agrees well with that made above and adds additional support to the idea that RhoA increases ENaC membrane levels and activity by affecting trafficking to the plasma membrane.

**Visualization and Quantification of ENaC Movement toward the Plasma Membrane**—The electrophysiology experiments described above, although informative, were indirect with respect to quantifying effects on trafficking for their end point measurements are channel activity. To directly visualize and quantify RhoA effects on ENaC trafficking toward the plasma membrane, we combined TIRF microscopy with FRAP in a modification of the approach first used by Axelrod and co-workers (25, 26) to investigate surface dynamics of biomolecules. With this approach, TIRF microscopy optically isolates fluorophore-tagged ENaC at the plasma membrane. This allows for signal selection from this pool of channels, as well as selective photobleaching of these channels. Recovery of fluorescence following photobleaching with TIRF illumination then allows quantification of the dynamics of channel trafficking toward the plasma membrane.

To our knowledge, this is the first application of TIRF-FRAP to the study of trafficking of an integral membrane. As such, we initially confirm selective photobleaching of ENaC at/near the plasma membrane and characterize ENaC movements as compared with those of a peripheral membrane protein at this cellular locale. The membrane maker eYFP-M was used as a reporter for the latter. The palmitoylation domain of neuromodulin localizes this fluorescent fusion protein to the inner leaflet of the plasma membrane.

The experiments in Fig. 2 demonstrate preferential photobleaching of ENaC in and near the plasma membrane abutting the coverglass versus total cellular pools of the channel with TIRF illumination. Shown in Fig. 2A are fluorescence micrographs of a cell expressing eYFP-ENaC (all three subunits tagged) before (left) and after (right) photobleaching with TIRF
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FIGURE 3. ENaC movement near the plasma membrane is restricted. A, red, green, and blue circles show the xy movement of typical circular clusters containing eYFP-M and eYFP-ENaC (before and after photobleaching), respectively, over time within the field of illumination in transfected cells. Images were acquired every 1 min. Trajectories and length of movement are indicated by the connecting lines. Trajectories are for representative particles from at least four different cells. Thus, although shown in the same figure, relative starting point and direction of movement are independent from one particle to the next with many of these particles actually residing in distinct cells. B, summary plot of mean $d^2$ versus time for eYFP-M (red; $n = 6$) and eYFP-ENaC (blue; $n \geq 10$) clusters describing movement in the field of illumination. C, summary graph for eYFP-ENaC shown with an expanded scale.

The region of the cell in Fig. 2A overlaying the left of the nucleus with particularly strong signal in the epifluorescence image is likely the Golgi complex. With TIRF microscopy, the excitatory evanescent field decays exponentially from the coverglass over ~100 nm (22). As apparent in the TIRF images, the Golgi complex is not visible, indicating that it is removed enough from the interface of the coverglass and plasma membrane to be outside the evanescent field. One hallmark of ENaC as visualized with TIRF microscopy is also clear in these images. This channel routinely appears in discrete clusters when viewed with TIRF microscopy. These clusters had morphologies ranging from small spheres of 0.82 ± 0.07 μm in diameter to elongated tubular structures of several microns in length (see also Figs. 4A, 5A, and 10B). In contrast to ENaC, eYFP-M had a uniform distribution with a small minority of this protein localizing to spherical particles of 1.1 ± 0.11 μm diameter (see Figs. 4A and 5A). ENaC distribution at/near the membrane reflects localization of the channel to discrete membrane microdomains or vesicles residing just below or docking with the plasma membrane.

To better understand movement of the channel or vesicles containing the channel at the membrane, particle tracking was used to compare the two-dimensional movement within the evanescent field of illumination for spherical clusters containing ENaC and eYFP-M in COS-7 cells. As shown in Fig. 3A by the red circles, eYFP-M was freely mobile and readily moved within the field of illumination. In contrast, ENaC movement, as defined by the blue and green circles in Fig. 3A, was severely restricted. Because movement of eYFP-M appeared random, we tested whether this movement represented diffusion. Fig. 3B plots the square of the two-dimensional distance ($d^2$) covered by eYFP-M (red) and ENaC (blue; shown with an expanded scale in Fig. 3C) clusters versus time. These data points were fit with the diffusion equation (defined in “Materials and Methods”) with the distance covered by both eYFP-M and ENaC increasing linearly over time. The calculated diffusion constants for eYFP-M and ENaC are 0.16 ± 0.005 and 0.0047 ± 0.001 μm²/min, respectively. Thus, clusters of eYFP-M move fast and are freely “mobile” compared with ENaC clusters, which move much slower to the point of being “immobile,” vibrating within a microdomain or docking site or around a tethering site.

If eYFP-M has a uniform distribution and is freely mobile, then fluorescence recovery of this reporter following photobleaching with TIRF illumination should result primarily from the lateral diffusion of nonbleached reporter from adjacent plasma membrane not in contact with the coverglass. As shown in Fig. 4, this is what we observed. In contrast, if ENaC movement is restricted, then recovery after photobleaching cannot occur via lateral diffusion and must represent trafficking of the channel toward the membrane into the evanescent field of illumination. Fig. 4A shows fluorescence micrographs of representative COS-7 cells expressing the membrane marker eYFP-M (top) and eYFP-ENaC (bottom). Cells were visualized with TIRF microscopy. Cells are shown just prior to photobleaching with TIRF illumination should result primarily from the lateral diffusion of nonbleached reporter from adjacent plasma membrane not in contact with the coverglass. As shown in Fig. 4, this is what we observed. In contrast, if ENaC movement is restricted, then recovery after photobleaching cannot occur via lateral diffusion and must represent trafficking of the channel toward the membrane into the evanescent field of illumination. Fig. 4A shows fluorescence micrographs of representative COS-7 cells expressing the membrane marker eYFP-M (top) and eYFP-ENaC (bottom). Cells were visualized with TIRF microscopy. Cells are shown just prior to photobleaching with TIRF illumination (Fig. 4A, left), and 10 s (middle) and 30 min (right) after photobleaching. Fluorescence recovery after photobleaching is present in both cases; however, a clear difference is present where recovery of eYFP-M but not eYFP-ENaC is uniformly spread across the entire field. This difference is particularly apparent in the line scans shown in Fig. 4B. These line scans show the normalized (to peak values at each time point) fluorescence emissions from eYFP-M and eYFP-ENaC in the areas defined by the dashed red lines for the cells in Fig. 4A. (The supplemental movies show complete experiments following recovery of eYFP-M and eYFP-ENaC as measured with TIRF-FRAP.) Importantly, we find that lateral movement of ENaC newly arrived to the field of illumination following photobleaching is also restricted (Fig. 3A, blue circles).
To further test the fidelity of measuring ENaC trafficking with TIRF-FRAP, we hypothesized that disrupting cellular integrity would interrupt ENaC trafficking toward the membrane but have little effect on lateral diffusion of eYFP-M within the membrane. Similar to that done previously (27–29), we used exposure to low concentrations of digitonin to chemically destroy cellular integrity by permeabilizing the plasma membrane. Fig. 5A shows TIRF images of cells expressing eYFP-M (top) and eYFP-ENaC (bottom) before, immediately after, and 15 min after photobleaching. Prior to beginning these experiments, cells were exposed to digitonin. As clear from these representative images and from the time course and summary graphs in Fig. 5, B and C, permeabilization of the plasma membrane disrupted recovery of eYFP-ENaC via trafficking but not eYFP-M via lateral diffusion.

**RhoA Accelerates ENaC Trafficking toward the Plasma Membrane**—We now were in a position to use TIRF-FRAP to directly probe the mechanism of RhoA actions on ENaC. Fig. 6A shows the time course of ENaC recovery within the evanescent field containing the plasma membrane following photobleaching with TIRF illumination in the absence (boxes) and presence of RhoA (ovals). All three subunits were tagged for these experiments. As summarized in Fig. 6B, 30 min after photobleaching the relative level of ENaC recovery in the presence of RhoA G14V at 0.68 ± 0.08 was significantly greater than the 0.49 ± 0.04 in the absence of this small G protein. Moreover, the time constant describing ENaC movement toward the membrane within the first few minutes following photobleaching, as reported in Fig. 6C, trended toward being faster in the presence of RhoA compared with its absence (see also supplemental Fig. S1). Co-expression of RhoA with ENaC thus resulted in faster and greater recovery of channels reflecting accelerated movement in the presence of the small G protein. Experiments in supplemental Fig. S1, where only a single type of subunit within the ENaC complex is tagged with eYFP, demonstrates, as expected, that ENaC movement into the field of illumination in the absence and presence of RhoA is independent of the reporter (tagged) subunit.

Nonbleached ENaC moving into the field of illumination may be newly synthesized channel protein or channels previously residing in compartments not available to photobleaching with TIRF illumination. Such compartments include, but are not limited to, recycling endosomes, the Golgi complex and plasma membrane not abutting the coverglass. The observation that relative emissions from total cellular pools of eYFP-ENaC...
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10 s and 30 min after bleaching with TIRF illumination are $0.75 \pm 0.04$ and $0.77 \pm 0.07$ ($n = 5$, not shown), respectively, excluding newly synthesized channel protein as the source of this ENaC.

The initial observations suggesting that RhoA promotes ENaC trafficking toward the plasma membrane were from electrophysiology experiments showing this small G protein to have additive effects on ENaC activity with dominant-negative dynamin (see Fig. 1A). Because dominant-negative dynamin retards endocytic vesicle budding rather than facilitating movement toward the plasma membrane, TIRF-FRAP measuring the latter, should be little influenced by overexpressing DN-Dyn. Fig. 7A shows the time course of recovery of eYFP-ENaC following photobleaching with TIRF illumination in cells expressing the channel alone (black boxes) and the channel with DN-Dyn in the absence (red circles) and presence of RhoA$^{G14V}$ (green triangles). As summarized in Fig. 7B, DN-Dyn alone compared with its absence had little effect on ENaC recovery. In contrast, FRAP in cells expressing the channel plus RhoA$^{G14V}$ and DN-Dyn (0.80 ± 0.08) was significantly greater compared with channel alone and channel plus DN-Dyn. The small difference in FRAP for ENaC in the absence and presence of DN-Dyn of 0.49 ± 0.05 and 0.58 ± 0.07, respectively, reflects the underestimation of trafficking toward the membrane because of constant retrieval of newly arrived nonbleached eYFP-ENaC. This newly arrived, nonbleached eYFP-ENaC accounts for only a small portion of the total ENaC in the field of illumination with the majority of channel in this field, at least initially, having destroyed eYFP because of photobleaching. Another way of stating this is that measuring ENaC movement into the field of illumination in the presence of DN-Dyn actually provides a truer reading for loss of fluorescence from newly arrived channels because subsequent internalization is minimized.

If disrupting channel retrieval has little effect on direct measurements of ENaC trafficking toward the membrane, then promoting channel retrieval should also have little effect on this measurement when made at steady state. We tested this by overexpressing Nedd4-2 with eYFP-ENaC and following trafficking toward the plasma membrane with TIRF-FRAP. Nedd4-2 plays an integral role in internalization and movement of ENaC away from the plasma membrane (1, 2). Fig. 8A shows the time course of eYFP-ENaC recovery following photobleaching with TIRF illumination in cells expressing the channel plus Nedd4-2. This time course of recovery is similar to that established for the channel in the absence of Nedd4-2 (see Fig. 6A). As summarized in Fig. 8B, Nedd4-2 had little effect on the maximum amount and speed of ENaC movement toward the membrane. However, as summarized in Fig. 8C, Nedd4-2 decreased activity of the channel as measured with electrophysiology as expected. This decrease in activity reflects decreases in membrane ENaC levels resulting from facilitated retrieval rather than slowed movement toward the plasma membrane.

Because RhoA facilitated movement of ENaC toward the membrane and intervention with ENaC retrieval had little effect on movement toward the membrane, we wondered whether we could slow ENaC trafficking to the membrane by disrupting the endomembrane system. Other laboratories have used brefeldin A previously to study trafficking of proteins, including ENaC, to the plasma membrane (30). This compound disrupts the endomembrane system causing disarray in normal cellular trafficking (31). Fig. 9A compares the time course for ENaC recovery as measured with TIRF-FRAP in naive cells (squares) and those treated with brefeldin A (circles). As clear in this figure and summarized in Fig. 9C, brefeldin A abolished ENaC movement toward the membrane. A quite different observation, as expected, was made when following recovery of eYFP-M in cells treated with brefeldin A. Fig. 9, B and C, demonstrates that disrupting the endomembrane system had no effect on recovery of eYFP-M. This is so because eYFP-M recovery in contrast to that of ENaC is mediated by lateral diffusion. Thus, maneuvers that impinge upon retrieval have little effect on ENaC trafficking to the membrane as measured with TIRF-FRAP, but those known to disrupt the endomembrane system impede movement of this channel toward the membrane without influencing recovery of a protein that rescues via lateral diffusion. Treatment with brefeldin A, as shown in Fig. 9D, decreased ENaC activity measured with electrophysiology...
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FIGURE 8. Facilitating retrieval with exogenous Nedd4-2 decreases ENaC activity but not trafficking toward the membrane. A, time course of relative FRAP of eYFP-ENaC at the membrane in the presence of co-expression of Nedd4-2. All other conditions the same as in Fig. 6A. The dashed line indicates FRAP at t = 30 min for ENaC in the absence of Nedd4-2 as established in Fig. 6A. B, summary graph of fluorescence recovery in the plasma membrane 30 min after photobleaching in cells expressing eYFP-ENaC plus Nedd4-2 (left ordinate). Also shown is the time constant describing this recovery (right ordinate). C, summary graph of ENaC activity measured in whole-cell voltage clamp experiments from cells expressing the channel alone and the channel plus Nedd4-2. *, versus ENaC alone.

FIGURE 9. Disrupting the endomembrane system decreases ENaC activity and trafficking toward the plasma membrane without affecting recovery of eYFP-M. A, time course of relative FRAP of eYFP-ENaC at the membrane in the absence (squares) and presence (circles) of brefeldin A. All other conditions the same as in Fig. 6A. B, time course of relative FRAP for eYFP-M at the membrane in the absence (squares) and presence (circles) of brefeldin A. C, summary graph of fluorescence recovery in the plasma membrane 30 min after photobleaching in cells expressing eYFP-ENaC and eYFP-M in the absence and presence of brefeldin A. *, versus untreated cells. D, summary graph of ENaC activity measured in whole-cell voltage clamp experiments from cells expressing the channel in the absence and presence of pretreatment with brefeldin A. *, versus untreated cells.

We next followed ENaC trafficking toward the membrane with TIRF-FRAP in cells with disrupted microfilaments and microtubules. As shown by the representative fluorescence micrographs in the top row of Fig. 10B, treating cells with cytochalasin D to disrupt microfilaments had little effect on the pattern of ENaC expression at/near the plasma membrane with channels remaining in clusters (n = 8). Moreover, cytochalasin D had no effect on ENaC recovery following photobleaching. Confirmatory studies using latrunculin B to disrupt microfilaments yielded identical results (n = 7; data not shown). In contrast, disrupting microtubules with colchicine, as shown in Fig. 10B (bottom row; see also supplemental Fig. S2), led to marked reorganization of ENaC clusters at/near the membrane (in 22 of 28 cells). This was associated with significantly slowed channel trafficking toward the membrane with relative FRAP after 15 min decreasing from 0.46 ± 0.04 to 0.28 ± 0.03 (n = 4) in the presence of colchicine.

**DISCUSSION**

We used TIRF-FRAP in conjunction with electrophysiology to define the mechanism by which RhoA increases ENaC activity. TIRF-FRAP enables quantitative investigation of trafficking events near the plasma membrane. We find that RhoA increases ENaC trafficking toward the plasma membrane to increase channel activity. We discuss here the possible underlying cell biology and significance of this finding, as well as the technology and technical advances associated with this study.

**Technical Advances**—The TIRF-FRAP method used in this study to follow movement of ENaC near the plasma membrane is a modification of that first used by Axelrod and co-workers (25, 26, 32, 33) to study surface dynamics of biomolecules and neuropeptide release via secretory vesicles. The major differences between our study and these earlier studies are that we follow movement of an integral membrane protein and sample at a slower rate to capture slower developing trafficking events. To our knowledge, this is the first study using such an approach to quantify trafficking of an intrinsic membrane protein toward the membrane. The key features of TIRF-FRAP are that it offers high spatial resolution, focuses directly on channels at/near the plasma membrane, is amenable to single cell studies, and allows...
measurements of channel movement in real time. Compared with biochemical and microscopy approaches commonly used to assess movement and membrane levels of intrinsic membrane proteins, including membrane labeling, ligand binding, and light and electron microscopy studies, this fluorescence-based biophysical approach allows for paired experiments, and in general requires less material and offers greater temporal and, in many cases, spatial resolution. It also is readily quantifiable. Combining real time particle tracking in living cells with TIRF-FRAP, as done in Fig. 3, moreover provides detailed information about the dynamics of movement near the membrane. In comparison with other fluorescence imaging approaches, such as confocal imaging, TIRF-FRAP offers greater resolution in the z axis (perpendicular to the membrane), ~100 nm versus ~0.5 μm, and uses a limiting evanescent field to focus on near membrane signals rather than fluorescent reporters to define membrane boundaries. Defining membrane boundaries with the intrinsic biophysical properties of the measurement rather than a membrane marker decreases the possibility of misidentifying membrane limits. The technical developments presented here may be generally applicable to the study of movement of intrinsic membrane proteins near the plasma membrane and other cellular functions involving near membrane events.

Measuring ENaC Movement Near the Plasma Membrane—Within the very thin region probed by the evanescent field, ENaC concentrated into circular and elongated structures. This is in contrast to eYFP-M, which had a predominantly uniform distribution at the membrane with a minority of the protein concentrating into clusters. The identity of the structures containing ENaC is discussed below. Nevertheless, movement of ENaC at/near the membrane was severely restricted compared with the movement of eYFP-M. Indeed, over a given time period ENaC moved laterally within an area more than 30 times smaller than the area covered by eYFP-M. The lateral diffusion constants calculated for eYFP-M and ENaC, although slower, were within the magnitude of those previously reported for similar types of proteins within the plasma membrane and for secretory granules near the membrane (32–34). The diffusion constants reported in this study likely underestimate actual movement for measurements made at room temperature with a slow sampling rate, which was necessary to record the entire recovery period for ENaC following photobleaching. Although missing events because of the slow sampling rate prohibits a complete understanding of the dynamics of ENaC movement in this two-dimensional field, the marked difference in relative movement of ENaC versus eYFP-M is what is most important to this study and the understanding its provides regarding the actions of RhoA on ENaC trafficking.

Our results clearly demonstrate that ENaC compared with eYFP-M is relatively immobile in the axis parallel to the membrane abutting the coverglass. The difference in mobility and uniformity of expression within the region probed for eYFP-M and eYFP-ENaC aided us in interpreting TIRF-FRAP results. As a consequence of its high mobility, eYFP-M recovery following photobleaching resulted from lateral diffusion within the membrane with reporter invading the region of the cell membrane in contact with the coverglass from surrounding membrane areas not abutting the coverglass, which were not bleached with TIRF illumination. Thus, eYFP-M recovery was first apparent at the edges of the membrane within the region probed by the evanescent field. In contrast, eYFP-ENaC recovery was uniform. Following photobleaching, recovered fluorophore-tagged ENaC reappeared in clusters. Thus, recovery of ENaC represents movement of the channel toward the membrane. Recovery is not via lateral diffusion but rather through a distinct mechanism involving trafficking.

Here we report six independent experimental results demonstrating that TIRF-FRAP is a reliable means for measuring ENaC movement toward the membrane. The first as discussed above relates to the dynamics and pattern of recovery. The second are results from experiments where cellular integrity was chemically destroyed. Permeabilizing the cell with low concentrations of digitonin abolished eYFP-ENaC but not eYFP-M recovery. These experiments were designed to disrupt normal cellular ionic, pH, and energy gradients, in addition to other cellular requirements necessary for trafficking, without doing major damage to the membrane abutting the coverglass. In addition, measurement of eYFP-ENaC movement toward the plasma membrane with TIRF-FRAP was independent of suppressing and activating endocytosis with genetic tools. Finally, TIRF-FRAP measurements of ENaC movement were sensitive to pharmacological disruption of endomembrane trafficking and microtubule organization. Together, these experiments underscore the utility and fidelity of TIRF-FRAP for measuring integral membrane protein trafficking near the plasma membrane.

ENaC Near the Membrane Is Caged—The shape, size, and lack of mobility of the circular structures containing ENaC are consistent with them being exocytic vesicles near the plasma
membrane as defined previously with TIRF imaging (32, 33, 35). The lack of mobility of such vesicles is believed to arise from their being docked at the membrane or tethered just below the plasma membrane by the cytoskeleton. Our results suggest that microtubules rather than microfilaments are involved in this tethering (see below). An alternative is that these structures are microdomains within the plasma membrane enriched in the channel. The pattern and size of ENaC clusters, as well as lack of mobility, are all consistent with the contemporary understanding of the plasma membrane as being a partitioned fluid with the cytoskeleton anchoring integral membrane proteins to form a “picket fence” (reviewed in Ref. 34).

In addition to localizing in circular structures, ENaC also appeared in elongated tubular structures that formed an interlacing meshwork. The exact relation between circular and elongated tubular structures is not clear, and we observe both in the same cell before and after photobleaching channels at the membrane. As discussed further below, disrupting microtubule organization impeded movement of ENaC toward the membrane and dissolved the elongated structures and meshwork. It is possible that these elongated tubular structures then represent vesicles containing ENaC that decorate the cytoskeleton just under the plasma membrane. Alternatively, they could represent ENaC within the membrane constrained by an underlying cytoskeletal meshwork. Irrespective of the exact nature and relation of these structures containing ENaC, we know they are within or near the plasma membrane and that the shape of these structures and ENaC trafficking to them are sensitive to disruption of microtubules and endomembrane movement.

RhoA Promotes Movement of ENaC toward the Membrane—The physiological question asked in this study is, “does RhoA increase ENaC membrane levels and activity by increasing trafficking of the channel to the membrane?” The current electrophysiology and TIRF-FRAP results answer this question in the affirmative.

It currently is not clear whether RhoA-mediated ENaC trafficking is a constitutive pathway supporting default movement of the channel to the plasma membrane or whether this cascade is capable of responding to signaling input to dynamically increase channel activity and Na$^+$ reabsorption. We do know, however, that in renal epithelial cells avidly reabsorbing Na$^+$, inhibiting RhoA signaling decreases Na$^+$ transport (7). Moreover, eYFP-tagged ENaC expressed in renal epithelial cells collects at the luminal plasma in puncta visually identical to those influenced by RhoA in this study.  

Thus, RhoA-mediated ENaC trafficking to the membrane likely plays a physiological role in control of Na$^+$ transport in epithelia. Moreover, trafficking of this channel to the membrane likely involves cellular and cytoskeletal structures common to many cell types. As such, the RhoA-sensitive trafficking of ENaC monitored in this study could represent a general cellular mechanism controlling trafficking of ion channels and other integral membrane proteins to the membrane. Supporting this is the recent finding that the Rho-GTPase Rac1 promotes translocation and insertion of functional TRPC5 channels into the plasma membrane of neurons to increase activity of this channel (12).

RhoA and related small G proteins are recognized to play regulatory roles in both cytoskeletal rearrangement and vesicle trafficking (13, 36–39). Thus, the cytoskeleton is a likely target for RhoA to influence ENaC trafficking.

The Role of the Cytoskeleton—Our results are most consistent with microtubules playing a role in ENaC movement to the membrane and the corolling of these channels at the membrane or the tethering of vesicles near the membrane containing these channels. Indeed, disrupting microtubule organization slowed ENaC movement, as well as leading to reorganization of ENaC at/near the membrane. This reorganization included dissolving the meshwork of elongated structures containing the channel, aggregation of circular structures at/near the membrane, and a general blurring of emissions from ENaC at/near membrane. In addition, ENaC accumulated into large sub-membrane complexes following disruption of microtubules. These sub-membrane complexes likely represent fusion of endomembrane compartments. These results demonstrate that normal microtubule organization is necessary for ENaC trafficking to the membrane. This is significant considering that microtubules are important for vectorial delivery of apical targeted post-Golgi vesicles in epithelia known to contain this channel (reviewed in Ref. 40).

We speculate that RhoA signaling either directs ENaC to the microtubule allowing vesicles containing this channel to follow this track toward the membrane, or RhoA reorganizes microtubules to push ENaC containing vesicles toward the membrane. The difference between these scenarios is that with the former, RhoA has specific actions on the channel, and with the latter, RhoA has specific actions on microtubules with a secondary consequence being movement of ENaC. In addition to affecting movement of the channel, our results show that microtubules also play a role in the arrangement of ENaC at/near the membrane. Thus, the pattern of ENaC expression at/near the membrane is coupled to how the channel arrives at this locale or microtubules play two independent roles being involved in both the movement of the channel toward the membrane and the organization of the channel at/near the membrane. We suspect that the final arrangement of the channel at/near the membrane and its activity are coupled to how the channel arrives there with RhoA increasing movement toward the membrane. This interpretation, in general, agrees with the reports of others that cytoskeleton adapter proteins and proteins involved in vesicle movement influence ENaC activity (41, 42).

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